Thesis for the Master of Science Degree in Molecular Biology

Androgen and glucocorticoid receptor mediated changes in histone acetylation at the MMTV promoter

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ABBREVIATIONS

AF	Activation function
AP-1	Activator protein-1
AR	Androgen receptor
ARA	AR associated proteins
ARE	Androgen response element
BAF	BRG1 associated factor
CARM	Coactivator-associated arginine methyltransferase
CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
CRH	Corticotrophin-releasing hormone
DBD	DNA-binding domain
Dex	Dexamethasone
DHEA	Dehydroepiandrosterone
DHEA-S	DHEA-sulphate
DHT	Dihydrotestosterone
ER	Estrogen receptor
FLH2	Four and a half LIM domain protein
FLIP	Fluorescence loss in photobleaching
FSH	Follicle stimulating hormone
GFP	Green fluorescent protein
GnRH	Gonadotropin releasing hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HAT	Histone acetyltransferase
HBO1	Human origin recognition complex interacting protein 1
HDAC	Histone deacetylase
HDACi	HDAC inhibitor
HMT	Histone methyltransferase
HRE	Hormone response element
HSP	Heat-shock protein
JMJ	Jumonji
JMJD2A	JMJA domain-containing protein
JMJD2C	JMJC domain-containing protein
KLK	Kallikrein
LBD	Ligand binding domain
LH	Luteinizing hormone
LHRH	LH-releasing hormone
LSD1	Lysine specific-demethylase 1
LTR	Long terminal repeat
MAPK	Mitogen activated protein kinase
MMTV	Mouse mammary tumor virus
LHRH	LH-releasing hormone
LSD1	Lysine specific-demethylase 1
LTR	Long terminal repeat
MAPK	Mitogen activated protein kinase
MMTV	Mouse mammary tumor virus
MR	Mineralocorticoid receptor
NCoR	Nuclear receptor corepressor
NES	Nuclear export signal
NFκB	Nuclear factor κB
NLS	Nuclear localization signal
NR	Nuclear receptor
NTD	N-terminal domain
P/CAF	p300/CBP-associated factor
PPAR	Peroxisome proliferator activated receptor
PR	Progesterone receptor
PRMT	Protein arginine methyltransferase
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
R1881	Methyltrienolone
RAR	all-*trans* Retinoic acid
RXR	9-*cis* Retinoic acid
SHBG	Sex hormone binding globulin
SMRT	Silencing mediator of retinoid and thyroid hormone receptor
SRC	Steroid receptor coactivator
STAT5	Signal transducer and activator of transcription 5
TAF	TATA binding protein-associated factor
Tip60	Tat interactive protein 60
TR	Thyroid receptor
TRAP	TR-associated protein
TSA	Trichostatin A
TST	Testosterone
VDR	Vitamine D3 receptor

GENERAL INTRODUCTION

1. Androgens

Androgens belong to a group of chemically related male sex hormones that are derived from cholesterol. They are required for the normal development of the penis, scrotum, testicles and the secondary characteristics of the male body as well as for the growth and development of prostate. In addition, androgens are implicated in the initiation and progression of prostate cancer.

Androgens are produced by the Leydig cells in the testis (90%) and by the adrenal cortex, a small gland located above the kidney. Testosterone (TST), of which more than 95% is secreted from the testis, is the major circulating androgen in men. The adrenal cortex and the testis also secrete other androgens, mainly dehydro-epiandrosterone (DHEA), DHEA sulphate (DHEA-S) and androstenedione. These hormones have only weak androgenic activity but they are important substrates for extragonadal synthesis of sex steroids (Labrie et al., 2001; Riggs et al., 2002). Secretion of testosterone is regulated by the hypothalamic-pituitary-testicular axis. The hypothalamus secretes locally acting luteinizing hormone-releasing hormone (LHRH), also known as gonadotropin-releasing hormone (GnRH), and corticotrophin-releasing hormone (CRH), that act on the pituitary gland. In response to these hormones, pituitary secretes luteinizing hormone (LH), follicle stimulating hormone (FSH) and adrenocorticotrophin (ACTH) that enter the circulation and affect the testis and adrenal glands. While LH acts on the Leydig cells to stimulate production of testosterone, ACTH stimulates production of adrenal androgens that are converted into testosterone. When testosterone levels in the bloodstream rise, the hypothalamus reduces the secretion of LHRH, which inhibits the secretion of LH from the pituitary gland and further reduces testosterone secretion. Thus, testosterone controls its own release through a negative feedback on the hypothalamic-pituitary-testicular axis.

In the bloodstream, testosterone circulates bound to one of two proteins, either sex hormone binding globulin (SHBG) or albumin. A small percentage of testosterone, approximately 2%, remains in a free, unbound form. While free testosterone and testosterone dissociated from albumin can enter the cell passively by diffusion, SHGB bound testosterone is transported into the cell actively through the membrane receptor (Rosner et al., 1999) (see Figure 3). In certain tissues, including the prostate, testosterone functions as a prohormone, where it is irreversibly converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. The biological functions of androgens are mediated through the androgen receptor (AR). This protein binds both testosterone and DHT, although it has a much higher affinity for the latter. In contrast to testosterone, DHT dissociates more slowly from AR and its binding induces a change in receptor conformation that is more resistant to degradation (Heinlein and Chang, 2004).

2. The Androgen Receptor

AR belongs to a superfamily of proteins that are referred to as nuclear receptors (NRs). This superfamily of structurally conserved, ligand-dependent transcription factors comprises more than 150 members that most likely evolved from a common ancestor (Escriva et al., 2000). Phylogenetic analysis has identified three major subfamilies within this superfamily, based on their ligand-binding and DNA-binding properties. AR, together with the estrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) belong to the classical steroid receptor subfamily. These NRs undergo nuclear translocation upon ligand activation and usually bind as homodimers to inverted repeat DNA half sites. A second subfamily of NRs includes receptors for thyroid hormone (TR), vitamin D3 (VDR), 9-*cis* retinoic acid (RXR), all-*trans* retinoic acid (RAR), and peroxisome proliferators (PPAR). This group of NRs is retained in the nucleus and usually binds to direct DNA repeats regardless of the presence of ligand. In addition, these

receptors exhibit promiscuous dimerization patterns, many involving heterodimerization with RXR. The majority of NRs identified to date form a third subfamily, so-called orphan receptors, which share a close structural relationship with receptors for known hormones but have no known ligands. Although most of them bind DNA as homodimers on direct repeats, some interact with RXRs while others bind as monomers to half-site sequences (Wilson et al., 1993; Perlmann and Jansson, 1995).

2.1 Structural features of AR

The AR gene is a single-copy gene located on the long (q) arm of the X chromosome between positions 11.2 and 12 (see Figure 1). It spans approximately 90 kilobases of genomic DNA. The coding region of the AR gene consist of eight exons separated by seven introns and encodes a polypeptide product of around 910-919 amino acids that corresponds to a 110 kDa protein.

2.1.1 Domain structure

Like other members of the NR superfamily, the AR contains distinct structural and functional domains that are conserved among the nuclear receptor family members: an N-terminal transactivation domain (NTD or A/B), a DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD).

The NTD or A/B domain

The N-terminal domain is the most variable both in size and sequence between NRs. In the case of AR, this domain is encoded by exon 1 that comprises more than half of the molecule (ca. 559 amino acids). It is involved in the transcriptional activation of target genes and contains a ligand-independent transactivation region, known as activation function 1 (AF-1). AF-1 is important for functioning of the full-length AR as its amino acid substitutions have been identified in patients suffering from androgen insensitivity and oligospermia (Gottlieb et al., 1999; Hiort et al., 2000). The NTD also contains three polymorphic direct repeats of glutamine (Glu), proline (Pro),

and glycine (Gly). Several studies have suggested that the change in the size of glutamine/glycine repeats alters the function of AR. The expansion of the size of the polyglutamine segment results in decreased AR transcriptional activity and is related to impaired spermatogenesis, infertility (Tut et al., 1997) and the spinal and bulbar muscular atrophy (Kennedy's disease) (La Spada et al., 1991), whilst shorter glutamine and/or glycine repeats may be related to prostate cancer incidence (Edwards et al., 1999; Wang et al., 2004). In addition, the androgen receptor NTD domain contains two motifs that contribute to intramolecular interactions with the LBD (He et al., 2000).

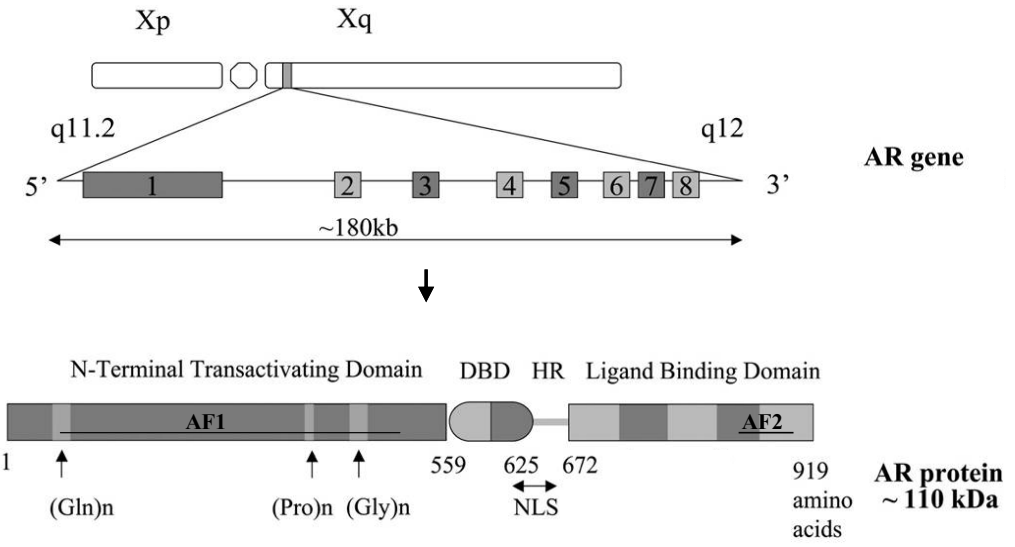


Figure 1. Schematic presentation of the AR gene and protein structure

AR is coded by a 180-kb gene located on the long arm of the X chromosome (11q11.2). The gene has eight exons (boxes) and seven introns (lines). After transcriptional processing, mRNA is translated into a 919-amino acid-long protein. A number of functional domains are recognized in AR protein: The N-terminal transactivation domain with indicated positions of glutamine (Gln), proline (Pro), glycine (Gly) repeats (arrows) and transactivation function AF1 (line); the central DNA binding domain (DBD); the hinge region (HR) and the nuclear localization signal (NLS); and the C-terminal ligand binding domain with the ligand dependent transactivation function AF-2. Figure modified with permission from The Journal of Clinical Endocrinology & Metabolism (Litvinov et al., 2003); Courtesy of John T. Isaacs, Ph.D.

The DBD domain

The DBD domain, encoded by exons 2 and 3, is the best conserved domain among the members of the nuclear receptor superfamily. It is characterized by a high content of basic amino acids and by nine invariant cysteine residues, of which eight are implicated in the formation of two zinc finger motifs. The N-terminal located zinc finger interacts directly with hormone response elements of target genes in the major groove of the DNA. The ability to determine the specificity of AR interaction with DNA resides in three amino acids [Gly; Ser; Val], located in the proximal box (P-box) at the base of the first zinc finger (Freedman, 1992). The second zinc finger helps to stabilize DNA receptor interaction and contains a five amino acid-long distal box (D-box), which participates in forming a dimerization interface for receptor monomers (Wong et al., 1993). Moreover, the AR DBD contains a non-classical nuclear export signal (NES) that mediates translocation from the nucleus (Black et al., 2001).

The hinge region

Located between the DBD and the LBD is a non-conserved hinge region, which can be considered as a flexible linker between the LBD and the rest of the receptor molecule. The hinge region is important for nuclear localization, containing a ligand-dependent bipartite nuclear localization signal (NLS) that also spans the C-terminus of the DBD. The nuclear targeting signal contains the consensus motif KxKK which is subject to acetylation, thus modulating AR function (Fu et al., 2000). In addition, the hinge region of all mammalian AR contains a PEST [Pro; Glu; Ser; Thr] rich sequence, which may function in proteasome mediated androgen receptor turnover (Sheflin et al., 2000).

The LBD domain

The second best conserved region of NRs is the C-terminal hormone binding domain. This domain is encoded by a portion of exon 4 and exons 5-8, and is responsible for the specific high-affinity ligand binding. The LBD is formed by 12 conserved α-

helixes and one β-sheet, together folded into a three-layered, antiparallel helical sandwich, creating a ligand-binding pocket for accommodation of ligand. Studies indicate that androgens interact with the LBD mainly through hydrophobic and hydrogen bonds (Matias et al., 2000). In addition to binding ligand, the LBD is also involved in dimerization, ligand-dependent coregulator recruitment and interaction of unliganded receptor with heat-shock protein (HSP) complexes. The LBD contains a ligand-dependent transactivation domain, known as activation function 2 (AF-2), which is also involved in interactions with coregulators (Slagsvold et al., 2000) and intramolecular interaction with the NTD (He et al., 2000).

2.1.2 Post-translational modifications

AR undergoes post-translational modifications, such as acetylation, ubiquitylation, sumoylation and phosphorylation (see Figure 2). These covalent changes may affect receptor stability, subcellular localisation and interactions with other proteins. Adding to the complexity, regulatory cross-talk between some of these modifications have been demonstrated (Fu et al., 2004; Rees et al., 2006).

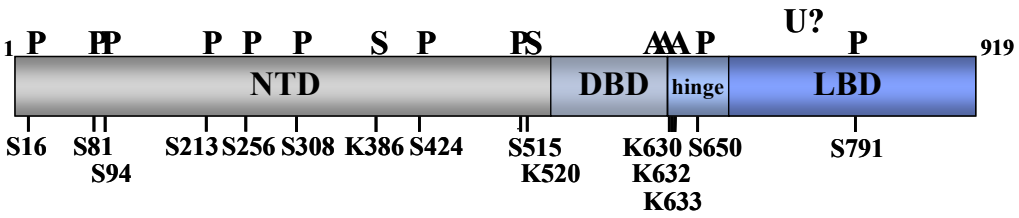


Figure 2. Schematic representation of the AR post-translational modification sites

P: phosphorylation, A: acetylation, U: ubiquitylation, S: sumoylation. U? indicates that the exact ubiquitilation site has not been identified.

AR is a phosphoprotein with multiple phosphorylation sites, mainly in the NTD. Most of these sites show increased phosphorylation in the presence of androgen, with the exception of Ser-94, which is constitutively phosphorylated. The kinases involved

in the phosphorylation of AR and the role of these modifications are still being debated. However, some studies suggest that MAPKs (Mitogen Activated Protein Kinases) and Akt play a role (Wen et al., 2000; Gioeli et al., 2006). Concerning acetylation, three lysines residues located in the hinge region at positions 630, 632 and 633 have been identified as acetylation sites. They play a role in the modulation of transcriptional activity by favouring nuclear translocation and by balancing coactivator and corepressor binding (Fu et al., 2002). Furthermore, cross-talk with phosphorylation has been suggested based on the fact that AR acetylation mutants change the pattern of AR phosphorylation (Fu et al., 2004). AR, similar to other steroid receptors, is subject to ubiquitylation. Several of the enzymes involved in this modification have been identified; however, the exact sites and functional relevance of this modification are still missing. AR is also postranslationally modified by sumoylation, namely at K386 and K520 in the NTD (Poukka et al., 2000). AR sumoylation is hormone dependent and results in mainly repressive effects. Sumoylation involves SUMO-1-conjugating enzyme Ubc9 that binds AR within the hinge region (Poukka et al., 1999), raising the possibility of cross-talk between acetylation and sumoylation.

2.2 AR transcriptional activation and regulation

In its unliganded state, AR exists in the cytoplasm in a complex with heat shock proteins (HSPs) such as Hsp90, Hsp70, and Hsp56. The complex is essential for the generation of a high-affinity, ligand-binding conformation of AR. Upon ligand binding, AR undergoes a conformational change, dissociates from HSPs and becomes hyperphosphorylated. HSP release unmasks the dimerization motifs and NLS site of the receptor that allows dimerization with another ligand-bound AR and nuclear translocation. The homodimer translocates to the nucleus where it binds androgen response elements (AREs) located in the promoters and/or enhancers of various androgen regulated genes (see Figure 3).

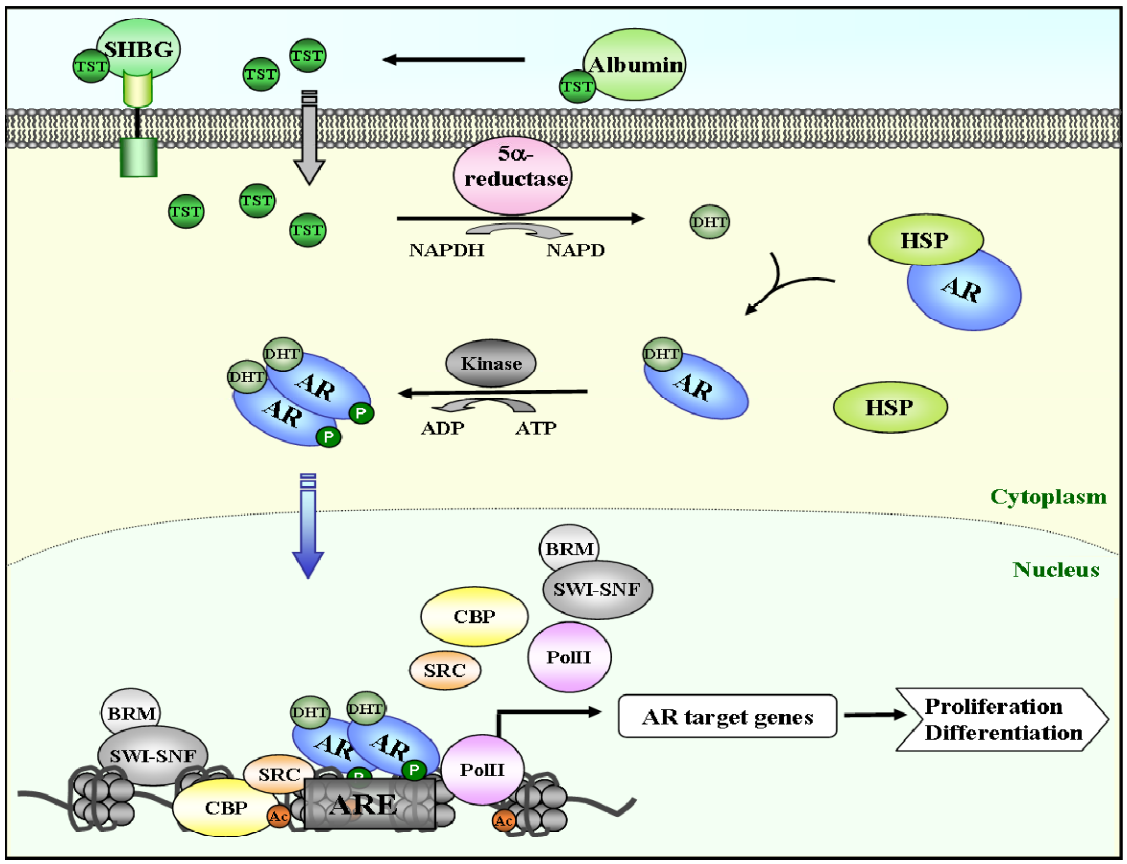


Figure 3. Schematic presentation of AR transcriptional activation

Testosterone (TST) diffuses into the cell or enters the cell through a sex hormone binding globulin (SHBG) receptor. TST is converted to 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase, and binds to the androgen receptor (AR). AR dissociates from the heat shock protein (HSP) complex, becomes phosphorylated and dimerize with another ligand-bound AR. The homodimer translocates to the nucleus where it binds to androgen response elements (AREs) of target genes, recruits coregulators and factors of the general transcriptional machinery. Transcription of AR target genes, mainly responsible for proliferation and differentiation, is induced. Figure reproduced with permission from (Klokk, 2007); Courtesy of Tove I. Klokk, Ph.D.

Although AR normally functions as a homodimer, it has been found to heterodimerize with other NRs including ER (Panet-Raymond et al., 2000), GR (Chen et al., 1997) and TR4 (Lee et al., 1999). The consensus response elements identified for AR are composed of two palindromic hexanucleotide half sites separated by a three-nucleotide spacer (AGAACAnnnTGTTCT). Despite the very different physiological effects of steroids, these AREs are also recognized by GR, PR

and MR. This paradox may be related to differential recruitment of coregulators upon the ligand-receptor interaction, although it remains to be further investigated. In addition, AREs composed of direct repeats and elements with modified site sequence have also been identified (Zhou et al., 1997; Geserick et al., 2005). After binding to ARE, AR initiates transcription of target genes through the recruitment of coregulator proteins, other transcription factors and factors of the general transcription apparatus. A schematic presentation of AR transcriptional activation is given in Figure 3.

2.2.1 AR coregulators

The transcriptional activity of AR, as well as other members of the NR superfamily, is influenced by coregulatory proteins. AR coregulators are generally defined as proteins that are recruited by AR and either enhance (coactivators) or reduce (corepressors) transactivation of target genes, without having significant effect on the basal transcription rate. In addition, coregulators do not typically possess DNA binding ability but contribute to AR mediated transcription through multiple mechanisms. Coregulators can influence AR transcription by acting with AR at the target gene promoter region to promote DNA occupancy, chromatin remodeling, histone modification, recruitment of general transcription factors associated with RNA polymerase II or by enabling the competency of the AR to direct target gene expression. The last mentioned can be achieved by modulating the appropriate folding of AR, ensuring its correct subcellular localization, facilitating ligand binding or intramolecular N/C interaction, thereby contributing to AR stability. In the last decade, an increasing number of proteins have been proposed to possess AR coactivating or corepressing characteristics (reviewed in Heinlein and Chang, 2002). A vast diversity of functions has been ascribed to these proteins, indicating that multiple cellular functions and signals regulate AR function. An overview of some well-known AR coregulators with their primary function is given in Table 1.

Table1. Overview of some well-known AR coregulators

Coregulator	coA/coR *	Direct/Indirect *	Function *
ARA24	coA	direct (NTD)	Signal transducer
ARA54	coA	direct (LBD)	Ubiquitination/proteasome
ARA55	coA	direct (LBD)	Focal adhesion
ARA70	coA	direct (LBD)	Nuclear receptor coregulator
ARA160	coA	direct (NTD)	Nuclear receptor coregulator
BAF57	coA	direct (DBD-hinge)	Chromatin remodeling complex
BRG1	coA	ND	Chromatin remodeling complex
hBRM	coA	ND	Chromatin remodeling complex
Calreticulin	coR	direct (DBD)	Chaperone
CARM1	coA	indirect	Histone methyltransferase
Caspase 8	coR	direct (NTD)	Regulator of apoptosis
CBP	coA	direct/indirect (DBD-hinge)	Histone acetyltransferase
Cyclin D1	coR	direct (hinge)	Cell cycle regulator
Filamin	coA	direct (DBD,LBD)	Cytoskeletal protein
FLH2	coA	direct (LBD)	Focal adhesion
HBO1	coR	direct (DBD,LBD)	Histone acetyltransferase
HDAC1	coR	direct (DBD,LBD)	Histone deacetylase
HDAC7	coR	direct	Histone deacetylase
HDACs(several)	coR	indirect	Histone deacetylase
Hsp40,70,90	coA	direct (LBD)	Chaperones
JHDM2A	coA	direct	Histone demethylase
JMJD2C	coA	direct	Histone demethylase
LSD1	coA	direct (NTD,DBD,LBD)	Histone demethylase
NCoR	coR	direct/indirect (LBD)	Nuclear receptor coregulator
p300	coA	direct/indirect (DBD-hinge)	Histone acetyltransferase
P/CAF	coA	direct/indirect (DBD-hinge)	Histone acetyltransferase
PRMT1	coA	indirect	Histone methyltransferase
PTEN	coR	direct (DBD)	Tumor suppressor
SIRT1	coR	direct (hinge)	Histone deacetylase
SMRT	coR	direct/indirect (NTD,LBD)	Nuclear receptor coregulator
SRG3	coA	direct (DBD-hinge)	Chromatin remodeling complex
SRC1	coA	direct (NTD,LBD)	Scaffold protein, HAT activity
SRC2	coA	direct (NTD,LBD)	Scaffold protein
SRC3	coA	direct (LBD)	Scaffold protein, HAT activity
Tip60	coA	direct (hinge-LBD)	Histone acetyltransferase
Trap/Mediator	coA	direct	Nuclear receptor coregulator

coA/coR-coactivator/corepressor; Direct/Indirect-direct or indirect association with the AR; ND-not determined; HAT-histone acetyltransferase; NTD- binding to N-terminal domain of AR; DBD-binding to DNA binding domain of AR; LBD- binding to ligand binding domain of AR. * As reviewed in (Heinlein and Chang, 2002; Wang et al., 2005a)

Coactivators

On a growing list of coactivators that regulate AR are the well studied coactivators of members of the p160 family of steroid receptor coactivator (SRC) family [SRC-1, SRC-2 and SRC-3], p300, the p300 homologue CREB binding protein (CBP), p300/CBP-associated factor (P/CAF), as well as Tat interactive protein 60 kDa (Tip60). In addition, protein arginine methyltransferases (PRMTs) such as coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1) have also been shown to play a role in AR transactivation. The majority of these coactivators possesses histone acetyl transferase (HAT) or methyltransferase (HMT) activity and is believed to act mainly through histone acetylation or methylation, thus modifying chromatin structure. In addition to their effects on histones, some can act through functional modification of proteins such as transcription factors and coregulators. Noteworthy in this regard is the fact that AR itself is acetylated by p300, P/CAF and Tip60 at three lysine residues in its hinge region (Fu et al., 2000; Gaughan et al., 2002). The concept that demethylation of histones could be also involved in transcriptional activation by the AR has emerged only recently. Since then, lysine specific-demethylase 1 (LSD1), the Jumonji A (JMJA) domain-containing protein JMJD2A, and the Jumonji C (JMJC) domain-containing protein JMJD2C, that demethylate lysine 9 on histone 3, have been shown to interact with and function as coactivators for AR (Metzger et al., 2005; Yamane et al., 2006; Wissmann et al., 2007). Several AR coactivators have also been identified as components of the ATP-dependent chromatin remodeling complex SWI/SNF, including the ATPases BRG1 and hBRM (Marshall et al., 2003), BAF57 (Link et al., 2005) and SRG3 (Hong et al., 2005). The recruitment of these proteins to the AR transcriptional complex is consistent with altered DNA topology following exposure to androgens. Another type of coactivator complex, that enhances ligand-dependent AR activity, is the multimeric thyroid hormone receptor (TR)-associated protein (TRAP)-mediator complex (Wang et al., 2002a), which appears to influence the basal transcription machinery, possibly through the direct recruitment of

RNA polymerase II. Transcriptional activity of the AR also relies on coactivators that bind to the AR-LBD or AR-NTD, facilitating AR stability or nuclear transport. Among these are AR associated proteins (ARAs) [ARA24, ARA54, ARA55, ARA70 and ARA160], the four and a half LIM domain protein (FHL2), filamin and Hsp40 (reviewed in Heinlein and Chang, 2002). In addition to the above mentioned coactivators, several other AR coactivators have been identified. However, the precise mechanisms by which many of these modulate AR activity are yet to be determined.

Corepressors

Most of the AR coregulators identified to date have been shown to enhance transcription of AR. Nonetheless, AR corepressors also play critical roles in regulating AR activity. Corepressor complexes often contain histone deacetylase (HDAC) activity that alters the acetylation state of histones, thereby regulating AR-mediated transcription. Cyclin D1 is an example of an AR interacting corepressor that functions through its ability to recruit HDACs and inhibit AR N/C interactions (reviewed in Burd et al., 2005). The two best characterized corepressors, the nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT), can directly associate with AR in the absence or in the presence of an agonist/antagonist and repress AR transactivation (Cheng et al., 2002; Liao et al., 2003). Even though both NCoR and SMRT recruit HDACs to target genes, evidence for a direct functional linkage between a specific HDAC and corepressor for AR is still missing. Thus, NCoR and SMRT may exert their repressive effects through other mechanisms, such as inhibition of AR N/C interaction or preventing coactivator binding. In contrast to the indirect recruitment of HDACs to the AR transcriptional complex, HDAC7, Sir2 and HDAC1 can interact directly with AR and repress its ligand-induced signaling (Gaughan et al., 2002; Fu et al., 2006; Karvonen et al., 2006). Similarly, calreticulin (Dedhar et al., 1994), the pro-apoptotic caspase-8 (Qi et al., 2007) and the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) tumor suppressor (Lin et al., 2004) interact directly with AR and repress its

transcriptional activity. However, these corepressors limit AR function by inhibiting AR nuclear translocation and/or DNA binding and not by direct repression of AR transcriptional activity. Another coregulator that associates with the AR and inhibits its activation is human origin recognition complex interacting protein (HBO1), a member of the MYST family (Sharma et al., 2000). The identification of HBO1 as an AR corepressor was surprising, as this protein contains HAT activity possessed by many coactivators. Nonetheless, the direct involvement of HBO1 enzymatic activity in its role as a corepressor has not yet been assessed. A number of other coregulators have been identified as AR corepressors. However, the mechanisms by which these corepressors inhibit AR transcativation remain to be elucidated.

2.2.2 AR and specific transcription factors

While there has been progress in describing the role of AR coregulators in AR dependent gene regulation, little is known about the roles of other DNA-binding transcription factors that may cooperate with AR in mediating androgen response. Over the last decade, numerous transcription factors have been shown to interact physically and functionally with the AR and regulate its transcription by different mechanisms. Some of these proteins interact directly with the AR and affect its ability to bind to AREs. One such example is dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X gene 1 (DAX-1) transcription factor that binds to the AR LBD and potently inhibits androgen-dependent transcriptional activation as well as the N/C terminal interaction (Holter et al., 2002). Other proteins can compete with the AR for coregulators that are present in limiting amounts in cells (Aarnisalo et al., 1998; Fronsdal et al., 1998). Moreover, some transcription factors, including FoxA1, Oct1 and GATA2 can bind DNA sequences in close proximity to AREs and cooperate to regulate AR target gene expression (Wang et al., 2007). The presence of these collaborating transcription factors may assist AR in binding to sites other than canonical AREs. Furthermore, some of these factors may function as pioneer factors that alter chromatin to permit AR binding. Overall, these studies

indicate that a hierarchical network of transcription factors with distinct functional roles can regulate distinct steps in AR dependent gene transcription.

3. Glucocorticoid Receptor

Glucocorticoids, a major subclass of steroid hormones, were originally named for their ability to influence glucose metabolism. During fasting, glucocorticoids help to maintain blood glucose levels by increasing for example gluconeogenesis, glycogen release, lipolysis and protein catabolism. In addition, glucocorticoids have effects on mood, cognitive functions and are important for inflammation and immune responses (reviewed in McMaster and Ray, 2007). Glucocorticoids are produced by the adrenal cortex and their biological effects are mediated via a 94-kDa intracellular protein, the glucocorticoid receptor (GR). GR is a member of the nuclear receptor superfamily and is one of the close relatives of AR. Like other steroid receptors, the GR consists of a variable N-terminal domain that also contains transactivation domain 1 (τ1), a central DNA binding domain with two zinc finger motifs, a hinge region, and a C-terminal hormone binding domain that harbors a second transactivation domain (τ2). GR exists in a number of splice variants that are expressed at different levels in different cell types and have differential activity on gene regulation (reviewed in Lu and Cidlowski, 2004). These include, for example, the conventional ligand binding GR termed GRα and a C-terminal variant that does not bind ligand, GRβ, which may have a dominant negative effect on GRα. In addition, it was reported that multiple proteins are translated from the GRα transcript, further increasing the diversity of GR protein expression (Lu and Cidlowski, 2005).

3.1 GR transcriptional activation and regulation

In its inactive, unliganded state GR is found predominantly in the cytoplasm complexed with HSPs, although a small fraction of GR/HSP complex may reside in the nucleus (Wikstrom et al., 1987), or recirculate to the nuclear compartment (Hache

et al., 1999). Independent of its intracellular localization, the main function of the GR/HSP complex is to keep the receptor protein in an inactive, ligand-activable state. Similar to AR, GR undergoes a conformational change upon ligand binding, dissociates from the HSP complex, becomes hyperphosphorylated, homodimerizes with another activated GR molecule and if cytoplasmic, translocates to the nucleus. In the nucleus, GR binds to glucocorticoid response elements (GREs) in promoters or enhancers of target genes, thereby inducing or repressing gene transcription (reviewed in Schoneveld et al., 2004). However, GR can also act as a monomer and modulate the transcriptional rates of non-GRE-containing genes by interacting with nuclear transcription factors, including activator protein-1 (AP-1), nuclear factor κB (NFκB) and signal transducer and activator of transcription 5 (STAT5) (reviewed in Bamberger et al., 1996). When associated with its response elements, GR initiates gene transcription through the recruitment of coregulatory complexes that modify and remodel chromatin, promoting a more open structure and further assembly of the basal transcriptional machinery (Baumann et al., 2001; McKenna and O'Malley, 2002; Kinyamu and Archer, 2004; O'Malley, 2004; Stavreva et al., 2004). Like other members of the steroid receptor subfamily, GR is subject to post-translational modifications, including phosphorylation, acetylation, ubiquitination and sumoylation (reviewed in Faus and Haendler, 2006). These may affect its transcriptional activity, stability and interactions with other receptors.

4. Nuclear receptor dynamics

4.1 Transcriptional action of NRs

AR and nuclear receptors in general mediate the action of their specific ligands through interaction with chromatin and protein-protein interactions with a variety of coregulators and basal transcription factors. The dynamic process by which the receptors recruit these factors to activate transcription was until recently poorly

understood. Currently, two opposing views exist for the development of transcriptional complexes on nuclear receptor regulated promoters: the classical view and the dynamic view, which are reviewed below.

4.1.1 The classic model

The classic model of nuclear receptor function proposes stable binding of the liganded receptor to the promoter. According to this view, nuclear receptors are stably associated with their recognition sites in promoters of target genes for as long as the ligand is present in the cellular milieu, serving as a platform for the sequential assembly of large transcriptional complexes (Shang et al., 2002). These complexes would have long residence times on the template, measured in minutes or hours. Indeed, AR activity was shown to involve sustained chromatin association with regulatory regions (Wang et al., 2005b). The occupancy of the AR-coactivator complex on regulatory regions increases gradually after androgen exposure, peaking at 16 hours and then gradually declining following longer stimulation. In contrast to AR, the ER transcription complex appears to cycle onto and off target promoters under continuous stimulation by estrogen, leading to a cyclical induction pattern with a periodicity of 40-60 minutes, at least on the well characterized ER target gene pS2 promoter (Shang et al., 2000). Even though the cyclic assembly of ER transcription complexes is a dynamic event, the central concept of a slow evolution of factor complexes (i.e. long term residency measured in 10s of minutes) remains. Evidence supporting this model has been obtained mainly from experiments based on chromatin immunoprecipitation (ChIP) studies. In the case of AR, ChIP studies have focused mainly on two AR target genes, prostate-specific antigen (PSA) and Kallikrein 2 (KLK2) (Kang et al., 2004; Wang et al., 2005b). Although the ChIP assay is a powerful tool to assess promoter occupancy and complex formation, it remains limited by the biochemical nature of the technique. Due to the difficulty in sample preparation and the need of fixation, ChIP cannot detect rapid protein movements. Furthermore, ChIP can assess the promoter occupancy only indirectly,

and thus it cannot confirm whether proteins are truly in a complex on a promoter. It can only show that they are somehow associated with the promoter sometime during the course of fixation. In addition, the results represent the promoter occupancy of an averaged cell population and cannot account for heterogenous cell responses. These features need to be considered in interpreting ChIP data.

4.1.2 The dynamic model

Recent studies making use of technological advances in live cell microscopy and genetically engineered cell lines challenged the classical view of stable template bound receptor complexes. This led to the proposal of an alternative, dynamic model for nuclear receptor action, called the "hit-and-run" model. According to this model, the receptor interacts transiently with the promoter, recruits other factors, and is itself dynamically displaced from its target sites (Hager et al., 2002). In contrast to the static view of receptor action, the residence time of NRs and interacting coregulators on the promoter would be measured in seconds, rather than minutes or hours. Evidence for this model was first provided by demonstration of the rapid exchange of green fluorescent protein (GFP)-tagged GR between chromatin and the nucleoplasmic compartment on a tandem array of mouse mammary tumor virus (MMTV) promoters, using fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching techniques (FLIP) (McNally et al., 2000).

Tagging the protein of interest with GFP and use of photobleaching techniques, such as FRAP and FLIP, allows a real time view of protein interactions with the chromatin template in live cells. In order to visualize and measure real-time mobility of NRs on their specific regulatory elements, the regulatory sites must be amplified in the chromosome. This was accomplished by constructing an artificial array with 200 copies of the steroid hormone receptor inducible MMTV promoter that contains HREs to which steroid receptors can bind directly (McNally et al., 2000) (see Figure 4).

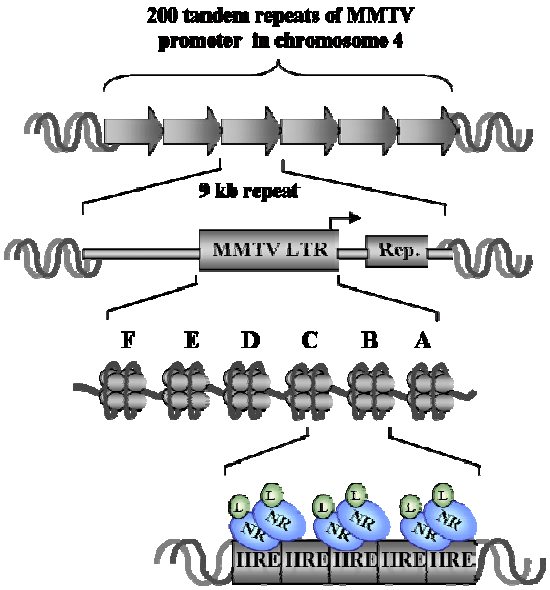


Figure 4. Schematic representation of the MMTV array

200 copies of a 9 kb repeat of the mouse mammary tumor virus (MMTV) promoter integrated into the host chromosome 4, creating a head-to-tail MMTV array. The MMTV promoter adopts a specific chromatin organization consisting of six positioned nucleosomes (A-F). Nucleosome B-C region of the MMTV long terminal repeat (LTR) contains hormone response elements (HREs) to which liganded nuclear receptors (NRs) can bind and regulate the transcription of a reporter gene (Rep). Figure reproduced with permission from (Klokk, 2007); Courtesy of Tove I. Klokk, Ph.D.

The MMTV array thus became a useful model system for the real-time study of protein-chromatin interaction dynamics, chromatin structure and promoter occupancy in relation to transcription. Similar high mobility of other transcription factors, including nuclear receptors as ER (Stenoien et al., 2001b), PR (Rayasam et al., 2005) and AR (Klokk et al., 2007), and transcription related factors (Stenoien et al., 2001b; Becker et al., 2002) have been demonstrated in the same or similar systems. Overall, from these studies a new insight into NR-mediated transcription has emerged that is based on highly dynamic interactions between the NR and the chromatin template. Schematic presentation of the hit-and-run model for AR action is presented in Figure 5.

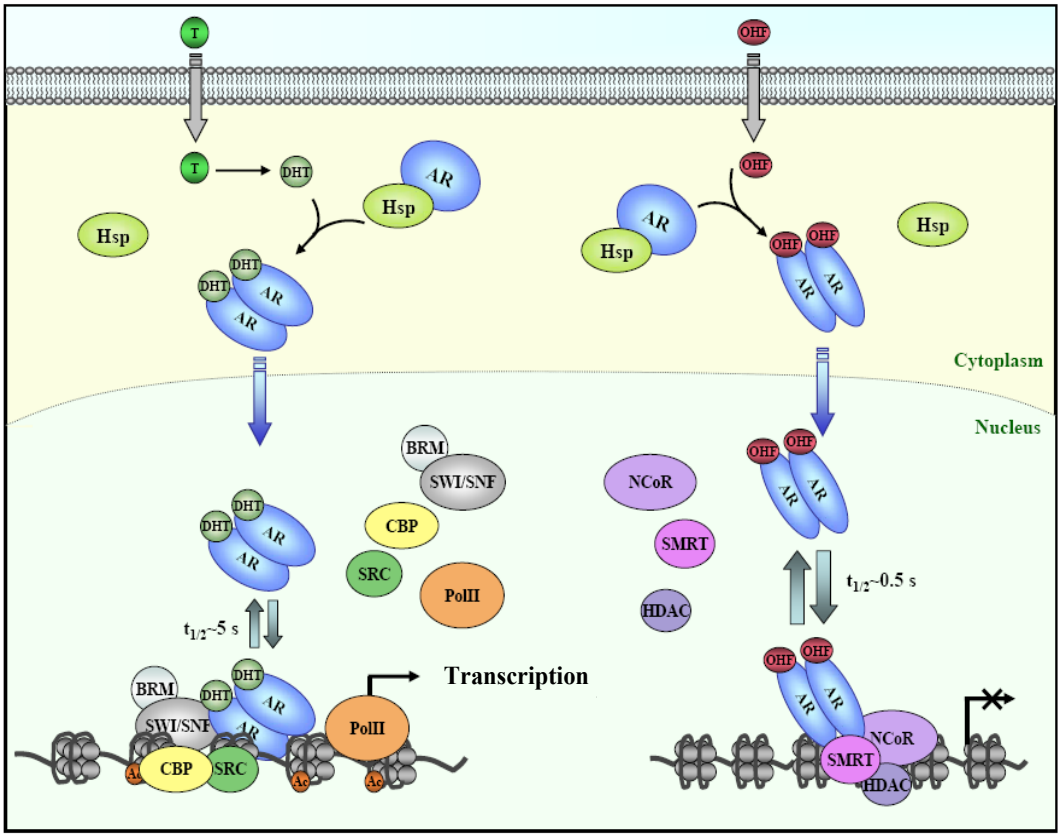


Figure 5. Hit-and-run model for AR action

AR bound to the agonist 5α-dihydrotestosterone (DHT) dissociates from the heat shock protein (Hsp) complex, translocates to the nucleus where it binds to androgen response elements (AREs) of target genes. Coregulator proteins are recruited, including chromatin remodeling complexes (SWI/SNF), coactivators (SRC), coactivators with histone acetyl transferase activity (CBP) and RNA polymerase II (PolII). The chromatin is remodeled, followed by association of a transcription initiation complex. AR is dynamically displaced (symbolized by the arrows) and shuttles between the chromatin-bound and free nucleoplasmic state with a half-maximal recovery time, $t_{1/2}$ of approximately 5 seconds. AR bound to the antagonist hydroxyflutamide (OHF), similar to agonist-bound AR, dissociates from (Hsps) and translocates to the nucleus. However, because of conformational changes induced by OHF, corepressors as NCoR and SMRT are recruited, leading to further recruitment of molecules, such as histone deacetylases (HDACs), which keep the chromatin in a compact state and inhibit the assembly of the transcription initiation complex. AR is displaced and shuttles between the chromatin-bound and free nucleoplasmic state with a half-maximal recovery time, $t_{1/2}$ of approximately 0.5 seconds. These rapid, stochastic interactions of AR with chromatin are influenced by the nature and outcome of its bound ligand. Figure adapted from (Kaarbo et al., 2007) with permission from authors.

4.1.3 The "static" versus "dynamic" view

The differences in nuclear receptor dynamics seen with live-cell approaches (time scale of seconds) and ChIP assays (time scale of minutes to hours) arise in part from the different time scales studied by these techniques. However, it has been proposed that these two apparently opposing views can be integrated into one consistent model (Hager et al., 2006; Metivier et al., 2006). The "return to template" model suggests that NRs exist in the nucleoplasmic space in various coregulator complexes that rapidly interact with their target regulatory site. Most of these rapid, transient binding events are stochastic and nonproductive, with only a small fraction resulting in template modification or the recruitment of alternate complexes. Thus, the promoter evolves through multiple specific events that modulate the receptor complex stability and initiation of transcription. In addition, both receptors and their coregulators are subject to modifications, which may alter the activity of the complex. Whereas FRAP experiments detect rapid and nonproductive binding of factors, ChIP assays would determine associations of productive complexes at a specific stage of promoter development, averaged over large number of cells, giving the impression of a statically bound complex. The rapid exchange of factors on promoters favors rapid activation and efficient promoter function and thus is very important for the cell to respond to changes in the environment. Alternatively, some genes that do not require a rapid response may still contain stable complexes. This suggests that individual promoters achieve transcription in different ways, depending on their architecture.

4.2 Mechanisms contributing to NR mobility

Despite the increasing number of transcription factors and their complexes that have been found to be highly mobile within the nucleus, the mechanisms involved in this behavior are yet not well understood. Current studies propose that several parameters affect and/or influence observed dynamic protein-chromatin interactions, including chromatin remodeling complexes, molecular chaperones, the proteasome degradation

apparatus, specific ligands, and modification of histones (reviewed in Hager et al., 2004) which are briefly reviewed below.

4.2.1 Ligand-specific dynamics of NRs

Live cell imaging experiments revealed that dynamics of steroid/nuclear receptors, such as GR (McNally et al., 2000), ER (Stenoien et al., 2001b), PR (Rayasam et al., 2005) and AR (Klokk et al., 2007), are strongly influenced by the nature of their ligands. For example, it has recently been shown in our laboratory that there is a significant difference between the dynamics of the AR-chromatin interactions in the presence of an agonist and that of an antagonist (anti-androgen) at an HRE in living cells (Klokk et al., 2007). Agonist-bound AR exhibits an approximately 10-fold slower FRAP recovery kinetics compared to antagonist–bound AR, suggesting that recovery kinetics is connected to transcriptional activity (see Figure 5). Furthermore, PR and GR have faster exchange rates compared to AR, indicating that receptors may use different mechanisms for interaction with their promoters. In general, the slower turnover rates of agonist/antagonist bound receptors might reflect the time needed for coregulator recruitment and assembly of the transcription machinery. In addition, mobilities of steroid receptors might also be affected by their ligand-induced conformational changes.

4.2.2 Chromatin remodeling and NR dynamics

The basic repeating unit of chromatin, the nucleosome, consists of 146 bp of DNA wrapped around an octamer of core histones, which is made of two copies each of the histone proteins H2A, H2B, H3, and H4. In addition to the four core histones, the linker histone H1, associates with DNA between nucleosomes and may facilitate the formation of larger chromatin fibers (higher order chromatin), leading to a fully condensed chromosome. The organization of DNA into chromatin restricts the access of promoters to regulatory proteins and the transcriptional machinery. This structural restriction of chromatin is overcome by two distinct classes of chromatin-remodeling

complexes: those that covalently modify histones (reviewed in Kouzarides, 2007) and those that rearrange the organization of the nucleosomes in the chromatin fiber in an ATP-dependent manner (Vignali et al., 2000; Chen et al., 2006). Recent data suggest that these complexes affect NR mobility and disassembly, in addition to their central role in remodeling (Fletcher et al., 2000; Nagaich et al., 2004; Metivier et al., 2006).

ATP-dependent chromatin remodeling complexes

ATP-dependent complexes use the energy derived from ATP hydrolysis to directly alter the position and/or stability of nucleosomes. They contain a core ATPase catalytic subunit that belongs to the Swi2/Snf2 superfamily of DNA helicases. Based on the identity of this subunit, five major families of ATP-dependent remodeling complexes have been described: SWI/SNF, ISWI, Mi-2/NuRD, INO80, and SWR1. Among them, the SWI/SNF family was the first identified and thus has been the best characterized. The human SWI/SNF complex contains one of two catalytic ATPase subunits, BRG1 or BRM, and several accessory BRG1-associated factors (BAFs). Although the BRG1 subunit of the SWI/SNF complex has been shown to be the primary component with regard to GR (Fryer and Archer, 1998) and PR (Mymryk and Archer, 1995) action, AR activity shows a strong dependence on the BRM subunit (Marshall et al., 2003). Results obtained over the last years demonstrate that the SWI/SNF remodeling complexes are also implicated in nuclear receptor dynamics. Using the template pull-down assays, both GR (Fletcher et al., 2002) and PR (Rayasam et al., 2005) were shown to be actively displaced from the nucleosome array during the process of chromatin remodeling, as a direct consequence of SWI/SNF action. In addition, recruitment of chromatin remodeling complexes by PR and GR were found to be ligand-dependent and the type of ligand associated with the PR, affected its displacement from chromatin during the process of remodeling. These results were more recently also extended to the AR (Klokk et al., 2007). The findings described above suggest that steroid receptors, in their liganded form, can recruit the ATPase subunit of the SWI/SNF complex, which is involved in their dynamic association with the chromatin template.

Histone modifications

The amino-terminal tails of histones are subject to a variety of reversible posttranslational modifications. At least eight distinct types of histone modifications have been identified to date, including acetylation, methylation, phosphorylation, and ubiquitination (Table 2). These alternations are directed by chromatin remodeling complexes that consist of the specific histone modifying enzymes (for example, kinases, histone acetyltransferases, methyltransferases, and ubiquitin ligases) (reviewed in Kouzarides, 2007).

Table 2. Modifications identified on histones

Modifications	Residues modified	Modifying enzymes
Acetylation	**K**-ac	Acetyltransferases (HATs)
Methylation (lysines)	**K**-me1 **K**-me2 **K**-me3	Lysine methyltransferases (HKMTs)
Methylation (arginines)	**R**-me1 **R**-me2a **R**-me3s	Arginine methyltransferases (PRMTs)
Phosphorylation	**S**-ph **T**-ph	Serine/Threonine kinases
Ubiquitylation	**K**-ub	Ubiquitilases
Sumoylation	**K**-su	Sumoylases
ADP ribosylation	**E**-ar	ADP-ribosyltransferases
Deimination	**R → Cit**	Peptidyl arginine deiminases
Proline isomerization	**P**-cis > **P**-trans	Proline isomerases

Overview of different modifications identified on histones. Modified residues: Lys (K), Arg (R), Ser (S), Thr (T), Glu (E), Pro (P) and Arg (R) to citrulline (C), and enzymes that direct each modification are shown. Modified from (Kouzarides, 2007).

Histone modifications may alter chromatin structure by influencing contacts between different histones in adjacent nucleosomes or the interaction of histones with DNA, or by recruitment of nonhistone proteins. However, current experimental evidence favors the view that histone modifications are epigenetic markers that facilitate the recruitment of chromatin binding proteins to dictate a distinct chromatin structure (histone code hypothesis) (Jenuwein and Allis, 2001). A number of proteins have been identified that are recruited to specific modifications and bind via specific domains. For example, acetylated histones are recognized by bromodomains (Yang,

2004), whereas chromodomains and PHD domains associate with methylated histones (Brehm et al., 2004), and 14-3-3 proteins bind phosphorylated histone H3 (Macdonald et al., 2005). The presence of such histone specific protein domains in some of ATP-dependent chromatin remodeling complexes indicates that there is a functional relationship between ATP-dependent chromatin remodeling complexes and histone modification. The most studied histone modifications are the acetylation and deacetylation of histone lysine residues which are reviewed below.

Histone acetylation

Histone acetylation is catalyzed by the enzymatic activities of histone acetyltransferases (HATs) that are divided into three main families: GNAT, MYST, and CBP/p300 (reviewed in Lee and Workman, 2007). Acetylation of histones neutralizes positively charged lysine side chains, which could weaken histone-DNA or nucleosome-nucleosome interactions, thereby creating a more open chromatin structure and enhance its accessibility to multiple transcription factors, such as the transcription complex. Indeed, acetylated chromatin has long been associated with transcriptionally active genes, with the rate of transcription correlating positively with the degree of histone H3 and H4 acetylation (Berger, 2002). In agreement with this many transcription coactivators that are recruited to target promoters by transcription activators, such as NRs, contain intrinsic HAT activity (Kuo and Allis, 1998). These include CBP/p300, P/CAF, TATA binding protein-associated factor (TAF) $_{II}$250, and the p160 family of coactivators. ChIP assays analyzing the timing of recruitment of different coregulators after ligand treatment have revealed that HAT containing complexes, similar to other coregulators, are recruited to target promoters in a dynamic manner and in a specific order (Metivier et al., 2006). The ordered recruitment of coactivators, changes in histone modifications and the recruitment of the transcription machinery, which leads to gene expression, were shown to correlate with cyclical recruitment of ER to the pS2 promoter. Additional transcription responses mediated by other NRs, such as AR and PR, have shown similar dynamic

temporal pattern of coactivator recruitment and histone modifications (Kang et al., 2004; Aoyagi and Archer, 2007). These findings indicate that association of HATs/HDACs complexes with target promoters and following changes in histone acetylation contribute to NR dynamics and promoter clearance.

Histone deacetylation and HDAC inhibitors

Acetylation of histones can be reversed by deacetylation that is catalysed by histone deacetylases (HDACs). Mammalian HDACs have been classified into four classes based on sequence homology to the yeast HDACs: class I (HDACs1-3 and HDAC8), class II (HDACs 4-7, HDAC9 and HDAC 10), class III (Sirt1-Sirt7), and class IV (HDAC11) which has properties of both class I and classII. Class III HDACs, so-called sirtuins, are homologs of yeast Sir2 and form a structurally distinct class of nicotinamine adenine dinucleotide (NAD)-dependent enzymes. HDACs remove the acetyl groups from histone lysine side chains, thus re-establishing the positive charge of histones and the less accessible form of chromatin that is commonly associated with transcriptional repression. In contrast to HATs, HDACs are often found as components of transcriptional repressor complexes such as NCoR and SMRT (Tsai and Fondell, 2004). Although HDACs have been generally correlated with gene repression, there are several examples where HDACs appear to be required for gene activation, thus functioning as coactivators (Berghagen et al., 2002; Wang et al., 2002b; Ferguson et al., 2003; Mulholland et al., 2003; Qiu et al., 2006).

The correct balance between HAT and HDAC activity plays an important regulatory role in gene expression. In addition to transcriptional regulation, HAT–HDAC interplay is also linked to other chromatin-associated processes such as replication, site-specific recombination and DNA repair, thereby playing a major role in modulating overall cellular fate (reviewed in Kouzarides, 2007). Increasing evidence indicates that alternations in HAT/ HDAC genes (such as translocation, amplification, over-expression or mutation) are connected to tumor growth and cancer (Cress and Seto, 2000). For example, histone deacetylation by HDACs may be a mechanism for

silencing some tumor suppressor genes responsible for cell progression, cell proliferation, differentiation and apoptosis. Inhibition of HDACs, and thereby activation of silenced genes, is therefore of interest in cancer therapy. To date, several natural and synthetic compounds with HDAC inhibitor (HDACi) activity have been identified. With a few exceptions, they can be divided into five main classes: hydroxamic acids, short-chain fatty acids, cyclic peptides, benzamides, and electrophilic ketones (reviewed in Minucci and Pelicci, 2006). Even though the action of HDACis in tumorigenesis has been explored and some of them are in clinical trials, several basic aspects are not yet fully understood and need further investigation.

5. Aim of the study

The main aim of this work was to study the molecular mechanisms by which AR regulates transcription. Previous studies suggested that the transcriptional activity of AR, as well as other steroid receptors, correlates with receptor mobility in the nucleus (Klokk et al., 2007). Furthermore, the dynamic behavior of AR was shown to be influenced by the nature of ligand (Klokk et al., 2007) and by changes in histone acetylation at the target promoter (results from Saatcioglu laboratory, unpublished data). However, other steroid receptors, such as GR, have been shown to have differential responses to changes in histone acetylation at the same promoter, compared to AR. To elucidate what links histone acetylation to the changes in AR dynamics, it is necessary to know the details of AR-chromatin interactions and the associated proteins at AR response elements under these conditions. The aim of this study was thus to examine in more detail local acetylation status of the MMTV promoter during AR-mediated transcriptional activation; this was compared with that of GR-mediated transactivation at the same response element.

MANUSCRIPT

Androgen and glucocorticoid receptor mediated changes in histone acetylation at the MMTV promoter

1. Summary

Post-translational modifications of histones play an important role in regulation of gene transcription. The most well studied histone modification is acetylation that is regulated by the enzymatic activities of histone acetyl transferases (HATs) and histone deacetylases (HDACs). Histone acetylation has generally been associated with transcriptional activation and deacetylation with repression. However, there are a number of genes for which activation is associated with deacetylation. Previous results from our laboratory show that increased histone acetylation induced by the HDAC inhibitor Trichostatin A (TSA) reduced androgen receptor (AR) mobility at the mouse mammary tumor virus (MMTV) promoter, concomitant with an increase in transcriptional activity. The effect of TSA was specific to AR as the dynamics and transcriptional activity of the glucocorticod receptor (GR), another ligand-regulated transcription factor of the steroid receptor family, was not affected by TSA. These data further demonstrated that histone acetylation does not always induce transcription, but is dependent on promoter and transcription factor context. In this study, the impact of TSA on the acetylation level of histones H3 and H4 at the MMTV promoter during AR- and GR-mediated transcriptional activation was investigated. Chromatin immunoprecipitation (ChIP) analysis revealed no significant change in histone acetylation at the MMTV promoter following TSA treatment, even though global levels of histone acetylation were greatly increased. Furthermore, global acetylation of histones occurred independently of the presence of androgen or

glucocorticoid. These results demonstrate that although TSA treatment induces a global increase in histone acetylation, specific locations of the genome, such as the MMTV promoter may be relatively unaffected. Interestingly, androgen treatment resulted in a decrease in the basal histone H3 acetylation level at the MMTV promoter. Preliminary studies suggest a different acetylation profile of histone H3 in the presence of GR compared to AR. However, additional studies are necessarry to reveal the details of histone acetylation during AR- and GR-mediated transcriptional activation.

2. Introduction

Androgens play a critical role in the development and maintenance of the male reproductive system and are involved in important physiological and pathological processes, such as normal prostate biology and prostate cancer (Jenster, 1999). The effects of androgens are mediated by the androgen receptor (AR), a ligand-regulated transcription factor that belongs to the nuclear receptor superfamily. Like other members of this family, AR is characterized by a structure composed of four distinct functional domains: an N-terminal transactivation domain (NTD) containing a ligand-independent activation function 1 (AF-1), a DNA-binding domain (DBD), a hinge region, and a ligand binding domain (LBD) possessing a ligand-dependent activation function 2 (AF-2). AR is a steroid hormone receptor which together with the closely related estrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR), form a subfamily of steroid hormone receptors. Upon ligand binding, steroid receptors change conformation, bind to their cognate hormone response elements (HREs) in promoters and/or enhancers of target genes and modulate transcription through the recruitment of chromatin modifying and remodelling complexes, coregulators, additional transcription factors as well as the components of the basal transcription machinery (Dilworth and Chambon, 2001; Hager, 2001; Marshall et al., 2003; Metivier et al., 2003; Wang et al., 2005a). The

classical view of steroid/nuclear receptor function suggests the static binding of the liganded receptors to regulatory elements in chromatin, which serves as a platform for the assembly of large transcriptional complexes (McKenna and O'Malley, 2002; Shang et al., 2002).

Advances in green fluorescent protein (GFP) technology and live-cell microscopy have led to the discovery of new principles for transcription factor action and the proposal of an alternative "hit-and-run" model (reviewed in Hager et al., 2006). According to this model, receptors interact only transiently with their HREs, recruit other factors and are dynamically displaced from the promoter. Dynamic movement on target promoters have been characterized for the steroid receptors GR (McNally et al., 2000), PR (Rayasam et al., 2005), ER (Stenoien et al., 2001a), and AR (Klokk et al., 2007), as well as for several other DNA binding proteins (Becker et al., 2002). In addition, various factors have been demonstrated to influence receptor mobility (reviewed in Hager et al., 2004). These include, among others, chromatin remodelling complexes, specific ligands and histone modifications. Indeed, it was revealed in our laboratory that dynamic interactions of AR are strongly dependent on the nature of the ligand, as agonist-bound AR had reduced mobility compared to antagonist-bound AR (Klokk et al., 2007). Moreover, longer residence time in the presence of agonist coincided with the recruitment of the ATPase BRM, chromatin remodeling and transcriptional activation. The involvement of specific ligands and the chromatin remodelling complex SWI/SNF in receptor mobility and transcriptional activation has also been demonstrated for other steroid receptors such as PR (Rayasam et al., 2005) and GR (Fletcher et al., 2002).

In addition to chromatin remodelling, the accessibility of promoters and transcriptional activity are also regulated by histone modifications (Berger, 2002). These are thought to contribute to the changes in histone-histone and histone-chromatin interactions that could lead to modulation of chromatin structure. Histone modifications can also act as signals for recruitment of additional chromatin-modifying factors, leading to changes in chromatin architecture and gene regulation

(Strahl and Allis, 2000; Jenuwein and Allis, 2001). In particular, acetylation of lysine residues within the N-terminal tails of histone proteins has been well studied in the context of gene regulation. Histone acetylation is regulated by the actions of histone acetyl transferases (HATs) and histone deacetylases (HDACs). The dynamic interplay between HATs and HDACs is thought to regulate histone acetylation at cellular and local promoter level (Struhl, 1998). Acetylation of histones has long been associated with transcriptional activation (Allfrey et al., 1964) and with an "open" and accessible chromatin conformation (Kuo and Allis, 1998; Verdone et al., 2005). In contrast, histone deacetylation is commonly correlated with gene repression and a more "closed", non-accessible form of chromatin. This view was solidified when several transcriptional coactivators, recruited to target genes, were identified to possess HAT activity, whereas many corepressor complexes were found in association with HDACs (Xu et al., 1999; Hu and Lazar, 2000; Tsai and Fondell, 2004).

However, a number of studies provide evidence that the relationship between histone acetylation and transcription is more complicated as transactivation of some promoters is associated with deacetylation. For instance, the treatment with histone deacetylase inhibitors (HDACis) that results in hyperacetylation of histones, showed inhibitory effects on steroid-inducible promoters, such as ER regulated ovalbumin promoter (McKnight et al., 1980) and mouse mammary tumor virus (MMTV) promoter regulated by GR (Bresnick et al., 1990; Mulholland et al., 2003).

The steroid-regulated MMTV promoter that assumes a well-defined chromatin structure when stably integrated into the host genome (Richard-Foy and Hager, 1987) has been a useful model system to study the relationship between chromatin structure, receptor dynamics and transcriptional activation. Previously, it has been shown that AR transcriptional activity on the MMTV promoter is induced by HDACi TSA (List et al., 1999). More recent results in our laboratory revealed that increased histone acetylation induced by the HDACis TSA and SAHA resulted in increased transcriptional activity of agonist bound AR, which correlated with reduced mobility

of AR at the MMTV promoter (unpublished data). The effect of HDACis on AR transcriptional activity and dynamics was receptor specific, as another member of the steroid hormone receptor family, GR, has been shown to have differential responses to changes in histone acetylation at the same promoter (unpublished data). It was previously reported that, in contrast to AR, TSA had inhibitory effect on GR activity on the MMTV promoter (Bresnick et al., 1990; List et al., 1999; Mulholland et al., 2003). In addition, results in our laboratory showed that transcriptional activity and mobility of agonist bound GR were not affected by the HDACis TSA or SAHA on the MMTV promoter, supporting the notion that reduced mobility of AR in response to HDACi was directly correlated with transcriptional activity. However, what links histone acetylation to the changes in AR dynamics and the molecular details underlying the differential response of AR and GR to HDAC inhibitors in the same promoter background is currently not clear. It was therefore of interest to examine the local acetylation status of the MMTV promoter in response to the HDAC inhibitor TSA during AR- and GR-mediated transcriptional activation that could possibly contribute to changes in receptor dynamics and transactivation potential.

3. Materials and Methods

3.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), DMEM-without Phenol Red, L-glutamine, Penicillin/Streptomycin and Trypsin/EDTA were purchased from BioWhittaker, Cambrex Bio Science and fetal calf serum (FCS) was purchased from PAA Laboratories GmbH. The following reagents were obtained from Sigma-Aldrich: Bovine serum albumin (BSA), magnesium chloride ($MgCl_2$), puromycin, dithiothreitol (DTT), β-glycerophosphate, sodiumortovanadate (Na_3VO_4), HEPES, Tween 20, leupeptin, phenylmethylsulphonyl-fluoride (PMSF), octyl phenoxy polyoxy ethanol (Triton X-100), sodium azide (NaN_3), trichostatin A (TSA),

dexamethasone (DEX), tetracycline, formaldehyde, phenol chloroform-isoamyl alcohol (25:24:1), lithium chloride (LiCl), sodium deoxycholate, sodium butyrate, yeast transfer ribonucleic acid (tRNA), NP-40/Igepal CA-630, anti-α-Tubulin mouse monoclonal antibody, horseradish peroxidase-conjugated (HRP) anti-rabbit IgG antibody and HRP-conjugated anti-mouse IgG antibody. Sodium chloride (NaCl), sodium hydroxide (NaOH) and ethylenediaminetetraacetic acid (EDTA) were purchased from BDH Chemicals Ltd. and sodium dodecyl sulphate (SDS) was obtained from Fluka Chemie GmbH. Methanol, trisaminomethane (Tris), hydrochloric acid (HCl) were obtained from VWR International, Inc. and glycine was from Duchefa Biochemie BV. Skim milk powder was from Acumedia Manufacturers, Inc. and salmon sperm DNA, Protein A Sepharose (liquid beads) and anti-GFP rabbit fraction antibody were purchased from Invitrogen. Lightcycler® 480 SYBR Green I Master mix, Lightcycler® Multiwell Plates 96, Protease inhibitor cocktail and proteinase K were obtained from Roche Diagnostics GmbH. Protein A Sepharose (powder beads) and ECL Western Blotting Analysis System were purchased from GE Healthcare Bio-Science. Anti-acetyl-histone H3, anti-acetyl-histone H4 and anti-AR rabbit polyclonal antibodies were obtained from Upstate Biotechnology, Inc. and another anti-AR rabbit polyclonal antibody (N-20) was from Santa Cruz Biotechnology, Inc. Anti-histone H3 rabbit polyclonal antibody was purchased from Cell Signaling Technology, Inc. and anti-GR mouse monoclonal antibody (BuGR2) was from Abcam, Ltd. Anti-GR rabbit polyclonal antibody was purchased from Affinity BioReagents, Inc. and ethanol was obtained from Arcus Kjemi AS. Precision Plus Protein™ Standards Dual Color, PVDF membrane and Bio-Rad protein assay were purchased from Bio-Rad Laboratories, Inc. Sodium acetate was from Merck Chemicals Ltd. and geneticin sulphate (G418) was from Gibco, Invitrogen Corporation. DyNazyme™ II DNA Polymerase with its buffer was obtained from Finnzymes Oy and deoxyribonucleosine-5'-triphosphates (dNTPs) and 2-log DNA ladder were purchased from New England BioLabs, Inc. MatTek cultureware 35mm glass bottom microwell dishes were obtained from MatTek Corporation and

MycoAlert® Mycoplasma Detection Kit was from Lonza Biologics, Inc. Methyltrienolone (R1881) was purchased from DuPont NEN Research Products and primers were manufactured by Sigma-Genosys.

3.2 Methods

3.2.1 Cell lines and Cell culture

The ell lines 3108 and 3617 are stably transfected derivates of the murine mammary adenocarcinoma cell line 3134 that contains 200 tandem repeats of a 9 kb element composed of the MMTV promoter followed by *ras* and BPV genes. These cell lines stably express GFP-tagged AR (3108) and GFP-tagged GR (3617),respectively, under the control of a tetracycline-off inducible system as previously described (McNally et al., 2000; Klokk et al., 2007). The passage number of both cell lines used in experiments was between 4 and 12. The cells were routinely maintained at 37ºC in a humidified 5% CO_2 and 95% air incubator in DMEM supplemented with 10% fetal calf serum (FCS), 5mg/ml penicillin-streptomycin, 2mM L-glutamine and 10µg/ml tetracycline (to suppress GFP-AR and GFP-GR expression). The 3108 cell line was additionally supplemented with 1mg/ml G418 and 0.55 µg/ml puromycin. The culture medium was changed every second day. The MycoAlert® Mycoplasma Detection Kit was used to test cells for mycoplasma contamination. For the experiments cells were plated in culture medium without G418 and puromycin at a density of 3×10^5 (3108) and 2×10^5 (3617) cells per 10 cm dish and grown in the absence of tetracycline for induction of GFP-AR and GFP-GR, respectively, if not indicated differently. After reaching 30% confluence, cells were washed with phosphate-buffered saline (PBS) and serum starved for 2 days in medium containing 10% charcoal treated (CT)-FCS to deplete the cells from steroids that could activate AR or GR. Prior to the experiments, cells were either left untreated or treated with TSA (100 nM) for 18 h, the synthetic androgen R1881 (10^{-8}M) for 1 h or the synthetic glucocorticoid DEX (10^{-7}M) for 30 min.

3.2.2 Protein extraction and Western analysis

Cells grown and starved in the presence or absence of tetracycline were harvested by scraping in PBS and collected by centrifugation. Whole cell extracts were prepared by resuspending the cell pellets in 200 µl lysis buffer containing 20 mM HEPES (pH 7.7), 300 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 M Na_3VO_4, 2µg/ml leupeptin and 0.5 M PMSF. After rotating for 2 h at 4ºC, supernatants were collected by centrifugation at 13,000 rpm for 20 min and the protein concentrations were determined by the Bio-Rad protein assay. For western analysis, proteins (100µg) were separated on 8% SDS-PAGE gels. The Precision Plus Protein^{TM} Standards Dual Color was used as a molecular weight marker. Proteins were then transferred overnight to PVDF membranes activated in methanol. Blotted membranes were blocked in 5% nonfat dry milk in PBS containing 0.1% Tween for 1 h, followed by incubation overnight at 4ºC with primary antibodies diluted in PBS-Tween containing different amounts of bovine serum albumin (BSA). Antibodies used were rabbit polyclonal anti-AR antibody (N-20, Santa Cruz) diluted in 3% BSA (1:500), rabbit IgG fraction anti-GFP antibody diluted in 5% BSA (1:1000), mouse monoclonal anti-GR antibody (BuGR2, AbCam) diluted in 3% BSA (1:400), and mouse monoclonal anti-α-tubulin antibody diluted in 0.5% BSA (1:4000). Membranes were washed with PBS-Tween and incubated for 1.5 h at room temperature with secondary horseradish peroxidase HRP-conjugated anti-rabbit (1:10000) or anti-mouse (1:5000) IgG antibodies diluted in PBS-Tween with 0.5% nonfat dry milk. All blots were visualized with the ECL Western Blotting Analysis System according to the manufacturer's instructions.

3.2.3 Live cell microscopy

For live cell imaging, cells were plated at a density of 7×10^4 (3108) and 4×10^4 (3617) cells per MatTek dish. The cells were grown in medium without tetracycline and starved as described above. Prior to the microscopy experiments, cells were washed with PBS and the regular starving medium was replaced with phenol red-free

medium supplemented with 10% CT-FCS to eliminate autofluorescence. The cells were then treated with R1881 (10^{-8}M) or DEX (10^{-7}M) for indicated time periods or left untreated. Microscopy was performed with an Olympus FluoView 1000 confocal laser scanning microscope, equipped with an incubator maintained at 37ºC, and images were acquired with a 60x1.3 numerical aperture oil immersion objective and a 488nm argon laser.

3.2.4 Histone extraction and Western analysis

Cells were plated, grown and starved under the same conditions as described previously. The cells were either left untreated or treated with R1881 (10^{-8}M), DEX (10^{-7}M), or TSA (100nM), alone or in combinations as indicated for the desired time periods. After washing with PBS, the cells were harvested by scraping in PBS and centrifugation. Harvested cells were washed twice with ice-cold PBS and resuspended in 500µl Triton extraction buffer (TEB) containing PBS with 0.5% Triton X-100, 2mM PMSF and 0.02% NaN_3. PBS was supplemented with 5mM sodium butyrate to retain levels of histone acetylation. The cells were lysed on ice for 10 min with gentle stirring followed by centrifugation at 2000 rpm for 10 min at 4ºC. After centrifugation, pelleted nuclei were washed in 250µl TEB and centrifuged as before. Nuclei were then resuspended in 150µl 0.2M HCl and lysed on a rotator at 4ºC overnight. The supernatant containing histones were collected after centrifugation at 2000 rpm for 10 min at 4ºC and protein concentrations were determined using the Bio-Rad protein assay, followed by Western analysis. 10 µg aliquots of each sample together with the Precision Plus Protein™ Standards Dual Color marker were resolved on 15% SDS-PAGE gels and transferred to methanol activated PVDF membranes overnight. Histone H3 and histone H4 acetylation were determined by blocking membranes in Tris-buffered saline (TBS) containing 0.1% Tween and 5% nonfat dry milk, and then probed with rabbit polyclonal anti-acetyl-histone H3 antibody (1:10000 dilution) or anti-acetyl-histone H4 antibody (1:5000 dilution) in TBS-Tween with 3% BSA overnight at 4ºC. Membranes were washed

and incubated for 1.5 h at room temperature with the secondary HRP-conjugated anti-rabbit IgG antibody diluted in TBS-Tween with 0.5% nonfat milk (1:20000). Protein bands were visualized using Western Blotting Analysis System. To verify equal loading of samples, membranes were stripped with 0.5M NaOH followed by incubation in TBS-Tween with 5% nonfat dry milk and reprobed with rabbit polyclonal anti-histone H3 antibody diluted in TBS-Tween with 3% BSA (1:2000). Following secondary antibody application visualization was performed as described above.

3.2.5 Chromatin Immunoprecipitation assay (ChIP)

Cells were plated, starved and pretreated with TSA, R1881or DEX for indicated time periods as described above. Three independent experiments were carried out for each cell line. Following treatment with hormone, cells were cross-linked with 1% formaldehyde in culture medium at room temperature for 10 min. The fixing process was stopped by the addition of glycine to a final concentration of 0.125M and incubation at room temperature for 5 min. After washing with PBS, the cells were harvested by scraping in ice-cold PBS supplemented with 1x protease inhibitor cocktail and centrifugation. The cell pellet was resuspended in 300µl lysis buffer [1% SDS, 10mM EDTA, 50mM Tris-HCl pH8, 1x protease inhibitor cocktail] and sonicated using Biorupter UCD-200 (Diagenode). During sonication, samples were kept in ice-cold water and sonication was performed on high setting using 21 pulses of 7 sec each, with a 7 sec rest between each pulse (in total 5 min). Cell lysates were then centrifuged at 13,000 rpm at 4ºC for 10 min to pellet the insoluble material. Supernatants were collected and for determination of DNA concentration and shearing efficiency, 15µl aliquots were removed prior to dilution in dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH8, 1x protease inhibitor cocktail] to a total volume of 3ml followed by immunoprecipitation.

DNA concentration measurement

4µl of each non-diluted sheared chromatin and 5µl proteinase K were added to 191µl lysis buffer supplemented with 40mM NaCl. The mixture was incubated at 65ºC for 1 h and DNA concentration was measured with Biophotometer spectrophotometer (Eppendorf) at 260 and 280nm.

Chromatin shearing

5µl of each non-diluted sheared chromatin was added to a mixture containing 3µl proteinase K, 184 µl lysis buffer and 8µl 5M NaCl. The mixture was incubated at 65ºC for 4 h or overnight and DNA was purified by extraction with phenol/chloroform, followed by addition of 0.1 volume of 3M sodium acetate and 2x volumes of ethanol and precipitation at -20ºC overnight. After 10 min centrifugation at 4ºC, the DNA pellet was washed once with 70% ethanol, dried and dissolved in 15µl double distilled water (ddH_2O). The size of the sheared DNA was determined on a 1% agarose gel. Sheared chromatin runs as a smear in the size range of ~100-1500 base pairs, where the major fraction is at about 300-800 base pairs.

Immunoprecipitation

Equal amounts of diluted sheared chromatin was adjusted to the volume of 3ml with the dilution buffer and precleared on a rotator for 2 h at 4ºC with 200µl salmon sperm DNA/protein-A sepharose slurry (50% v/v beads in TE buffer supplemented with 0.2mg salmon sperm DNA and 0.5mg BSA/ml) to avoid non-specific binding of protein-A sepharose. After preclearing, the beads were briefly centrifuged at 1000 rpm at 4ºC for 1 min, the supernatants were collected and 1/10 of each sample was stored at 4ºC as an input control. The supernatant fractions were then subjected to chromatin immunoprecipitation overnight at 4ºC with 8 µg of either anti-acetyl histone H3 or H4 antibodies, 2µg anti-AR antibody (N-20, Santa Cruz) and 10µg anti-GR antibody (Affinity BioReagents). A control without any antibody was included for assessing non-specific binding. Following incubation with antibody, 80µl of salmon sperm DNA/protein-A sepharose was added to each tube for 1 h with

rotation at 4ºC to isolate the immune complexes. The sepharose-antibody-chromatin complexes were then captured by centrifugation at 1000 rpm for 3 min at 4ºC and washed sequentially for 15 min (washes on a rotator platform, followed by centrifugation at 1000 rpm for 3 min), first in TSE I buffer [0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8, 150mM NaCl], TSE II buffer [TSE I with 500mM NaCl], buffer III [0.25M LiCl, 1% NP-40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris-HCl pH8], and then in TE [10mM Tris-HCl pH8, 1mM EDTA]. All steps starting with the harvesting of the cells to the elution step were done in the presence of 5mM sodium butyrate. Sepharose bound proteins were then eluted twice with 250µl elution buffer [1% SDS in ddH_2O] on a rotator at room temperature for 15 min and eluates were combined. The stored input samples were eluted with 200µl elution buffer [ddH_2O + 1% SDS in final solution]. Eluates were mixed with 20µl 5M NaCl, 10µl 0.5M EDTA, 20µl 1M Tris-HCl pH6.5 and 15.6 µg of proteinase K, and heated at 65ºC for at least 4 h to reverse the formaldehyde cross-linking between DNA and protein. DNA fragments were purified by phenol/chloroform extraction and precipitated with ethanol and 10µg carrier RNA (yeast tRNA) overnight at -20ºC. Following centrifugation, the DNA pellet was washed with 70% ethanol, dried and dissolved in 40µl ddH_2O for further analysis by PCR.

PCR analysis

Following purification, the DNA was subjected to PCR amplification using primers specific for nucleosome B region of the MMTV promoter. The corresponding PCR product was 120 base pairs. Primers used were: forward 5`-TTTCCATACCAAG-GAGGGGACAGTG-3` and reverse 5`-CTTACTTAAGCCTTGGGAACCGCAA-3`. With all experiments, negative (no template-ddH_2O) and positive (LTR plasmid) PCR controls were included. The PCR reaction was performed in a mixture (25µl), containing 2µl DNA, 10x reaction buffer, 0.4mM each of dATP, dTTP, dGTP and dCTP, 1.6mM $MgCl_2$, 1 unit of DyNazyme II DNA polymerase, and 0.4µM of each primer. The reaction was carried out in a Peltier Thermal Cycler (PTC-200; MJ Research) using the following amplification conditions: 5 min denaturation at 95ºC

followed by 30 cycles of denaturation at 95ºC for 30 sec, annealing at 63ºC for 1min and extension at 72ºC for 1 min. In the end, there was a final extension for 5 min at 72°C. The amplified DNA was analyzed on a 1.5% agarose gel.

3.2.6 Quantitative PCR (qPCR)

DNA isolated from precipitated chromatin was analysed by qPCR on a LightCycler® 480 (Roche Diagnostics) using SYBR Green as a DNA-specific binding dye and continuous fluorescence monitoring. The PCR reaction (10µl) contained 2x SYBR Green I Master mix, 2µl of DNA template and 0.5µl of each primer. The primers, positive and negative controls used were same as described in conventional PCR analysis. The PCR program was comprised of an initial denaturating step at 95ºC for 5 min followed by 45 cycles of denaturation (15sec at 95ºC), annealing (30sec at 63ºC) and extension (20sec at 72ºC). To avoid non-specific PCR by-products and detect presence of possible primer dimers, melting curve was analysed: 5sec at 95ºC, 1 min at 65ºC followed by continuous fluorescence measurement between 65ºC and 97ºC. The crossing points (Cp) values calculated by the LightCycler computer software were used for determination of the initial amount of DNA template.

Data analysis

The data analysis was performed using Microsoft Office Excel 2003 software. The qPCR signals derived from the ChIP samples were normalized by the signals derived from the input samples. The level of acetylation in the absence of ligand and TSA treatment and the level of receptor enrichment in the absence of ligand were set to 1. The data presented represent the average of three independent experiments. Statistical analysis was performed using the Student's two tailed t-test. Values of $p < 0.05$ were considered statistically significant.

4. Results

4.1 Tetracycline-regulated expression of GFP-AR and GFP-GR

In order to examine AR and GR mediated chromatin modifications of the MMTV promoter, the previously established 3108 and 3617 cell lines were used (McNally et al., 2000; Klokk et al., 2007). These cell lines contain an integrated tandem repeat of the MMTV promoter and express GFP-AR (3108) or GFP-GR (3617) in a stable and tetracycline repressible manner. Regulation of GFP-AR and GFP-GR expression in these cells by tetracycline was confirmed by Western analysis of total cell lysates, using antibodies against AR, GR and GFP. As can be seen in Figure 1, there was clear induction of GFP-AR (A) and GFP-GR (B) expression upon 72 h withdrawal of tetracycline from the medium, resulting in a band of expected size (~130 kDa).

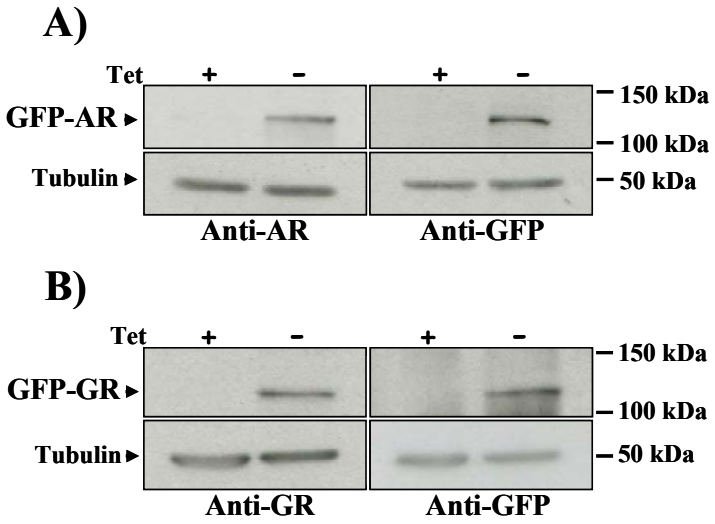


Figure 1. Tetracycline-regulated expression of GFP-AR and GFP-GR

3108 (A) and 3617 (B) cells were grown and starved, as described in Materials and Methods, in the presence (+) or absence (-) of tetracycline (Tet). Whole cell extracts were prepared and subjected to Western analysis with antibodies specific to AR (A, upper-left panel), GR (B, upper-left panel) and GFP (A-B, upper-right panels). Tubulin was used as a loading control (A-B, lower panels). Molecular weight markers are given to the right.

4.2 Ligand-dependent translocation of GFP-AR and GFP-GR to the nucleus and their recruitment to the MMTV array

The integrated MMTV array in the 3108 and 3617 cells contains approximately 800 to 1000 HREs, which act as binding sites for both AR and GR. This has enabled the visualization of the GFP tagged AR and GR when bound to their response elements in living cells. Previously it was demonstrated that GFP-AR and GFP-GR within these cell lines translocate to the nucleus and bind to the array upon ligand activation (McNally et al., 2000; Klokk et al., 2007). We have also confirmed the effect of synthetic androgen R1881 (methyltrienolone) and synthetic glucocorticoid Dexamethasone (Dex) on intracellular translocation of GFP-AR and GFP-GR by live cell confocal microscopy (Figure 2).

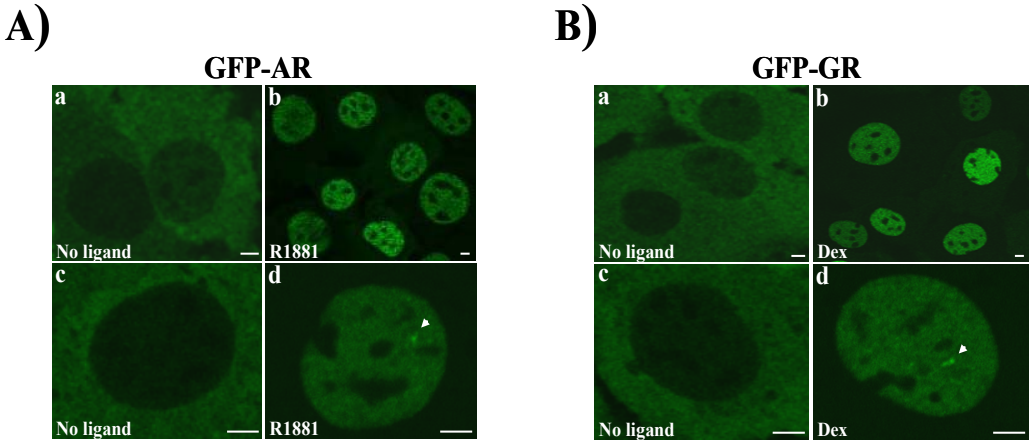


Figure 2. GFP-AR and GFP-GR translocate to the nucleus and bind to the MMTV array in response to their ligands

Intracellular localization of GFP-AR (A) and GFP-GR (B) followed by in vivo time-lapse confocal microscopy at 37°C. (A) 3108 cells expressing GFP-AR were left untreated (a and c) or were treated with the synthetic androgen R1881 (10^{-8} M) for 30 min (b and d). GFP-AR was completely translocated to the nucleus after 30 min in response to R1881 (b) and recruited to the MMTV array (d). No translocation and binding to the array was observed in the absence of R1881 (a and c). (B) 3617 cells expressing GFP-GR were treated with the synthetic glucocorticoid Dexamethasone (Dex) (10^{-7} M) for 30 min (b and d) or were left untreated (a and c). GFP-GR was completely translocated to the nucleus after 30 min in response to Dex (b) and recruited to the MMTV array (d). No translocation and binding to the array was observed in the absence of Dex (a and c). Arrows point the position of the array. Bar, 4µm.

Because the array in these cells exists as a unique, single amplified element on chromosome 4, a single bright GFP fluorescence signal was expected to be observed within the nucleus. In the absence of ligand, GFP-AR and GFP-GR were distributed predominantly in the cytoplasm and no significant binding to the MMTV array was observed (Figure 2Aa, 2Ac, 2Ba and 2Bc). The presence of agonists R1881 or Dex for 30 min caused complete translocation of AR or GR, respectively, to the nucleus (Figure 2Ab and 2Bb). Furthermore, a single bright fluorescent signal (Figure 2Ad and 2Bd), in addition to the diffuse nucleoplasmic GFP-AR and GFP-GR, was detected within the nucleus. These data confirm the intact nature of these cell lines as described in previous studies (McNally et al., 2000; Klokk et al., 2007).

4.3 HDAC inhibitor TSA induces global histone acetylation independently of the presence of hormone

HDAC inhibitors (HDACis) are generally used to manipulate the acetylation status of histones and to induce global histone hyperacetylation. Trichostatin A (TSA) is an efficient HDAC inhibitor (HDACi) and was shown to induce an increase in acetyl-histone H3 levels in 3108 and 3617 cells in a time-dependent manner (Saatcioglu laboratory, unpublished data). To assess whether exposure of 3108 and 3617 cells to TSA treatment give rise to expected increase in histone H3 and H4 acetylation, cells were left untreated or were treated with 100 nM TSA for 18 h. Moreover, in order to examine if the TSA-induced histone acetylation was dependent on the presence of hormone, 3108 and 3617 cells were additionally treated with R1881 or Dex, respectively, alone or were pretreated with TSA prior to ligand addition. The histone extracts were obtained and subjected to Western analysis using antisera specific for acetyl-histone H3 and acetyl-histone H4 (Figure 3).

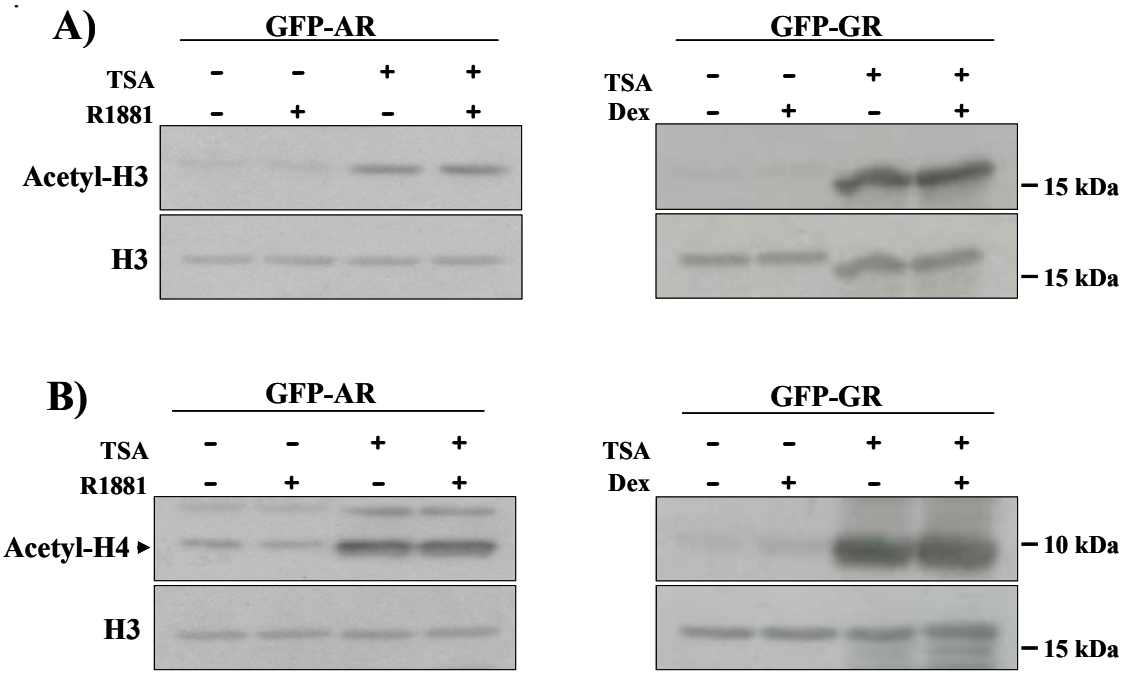


Figure 3. Acetylation of histone H3 and H4 in response to TSA and ligand activation

3108 and 3617 cells were treated with TSA (100nM) for 18 h, or R1881 (10^{-8} M) for 1h or Dex (10^{-7} M) for 30 min, alone or in combinations, or were left untreated. Histone extracts were obtained and subjected to Western analysis with antibodies specific to acetyl-histone H3 (A) or acetyl-histone H4 (B). Anti-histone H3 antibody was used as a loading control (lower panels A and B). Molecular weight markers are indicated to the right of the figure.

In the absence of TSA, both histones H3 (A) and H4 (B) exhibited low levels of acetylation that were strongly increased in response to TSA; total H3 levels were not affected. This confirmed the efficient inhibition of HDAC activity by TSA in 3108 and 3617 cells. Furthermore, addition of R1881 or Dex did not alter the histone acetylation profiles generated by treatment with TSA alone. Thus, exposure to TSA results in global increase of histone acetylation patterns, independently of the presence of hormone. The acetyl-histone H4 antiserum also detected additional non-specific band in 3108 cells at around 13 kDa (Figure 3B, left panel). This band shows similar response to TSA treatment as observed for acetylated histone H4 and might represent cross reaction with acetylated histone H2A (~14kDa). Alternatively, this

could be a proteolytic fragment of histone H4 produced during extract preparation. However, the nature of this band was not investigated further.

4.4 ChIP optimization

Having established that the cell lines we wished to use in this study behaved as expected, we set out to use chromatin immunoprecipitation (ChIP) in these cells to probe dynamic changes of protein interactions at the MMTV array. ChIP is a powerful method used to identify regions of the genome associated with specific proteins, histone modifications, chromatin remodeling and other chromatin-related processes. In this study, the ChIP assay was used to study histone acetylation profiles at the Nuc-B region of the MMTV promoter in 3108 and 3617 cells. In addition, association of AR and GR with HREs of the MMTV promoter were also assessed by this method. The ChIP assays were carried out according to the standard protocol that was previously established in our laboratory, as described in Materials and Methods. However, after performing some preliminary ChIP assays to optimize it for use in these experiments, we lost all ChIP signal which was weak to start with; therefore, we needed to do several rounds of troubleshooting.

To that end, the 3108 cells were left untreated or were treated with androgen R1881 and subjected to the ChIP assay according to our standard protocol (Figure 4). Because the resolution obtained by the ChIP procedure depends on the size of chromatin fragments, it was first important to check if the shearing conditions used in the protocol resulted in the expected DNA smear in the desired size range (~200~1000 bp). As seen in Figure 4A, the major fraction of sheared DNA had optimal length of ~300~800 bp, similar to that detected by previous ChIP experiments performed in our laboratory (data not shown). Having established this, ChIP assay was processed to the end with the AR antibody which resulted in no ChIP based signal (Figure 4B).

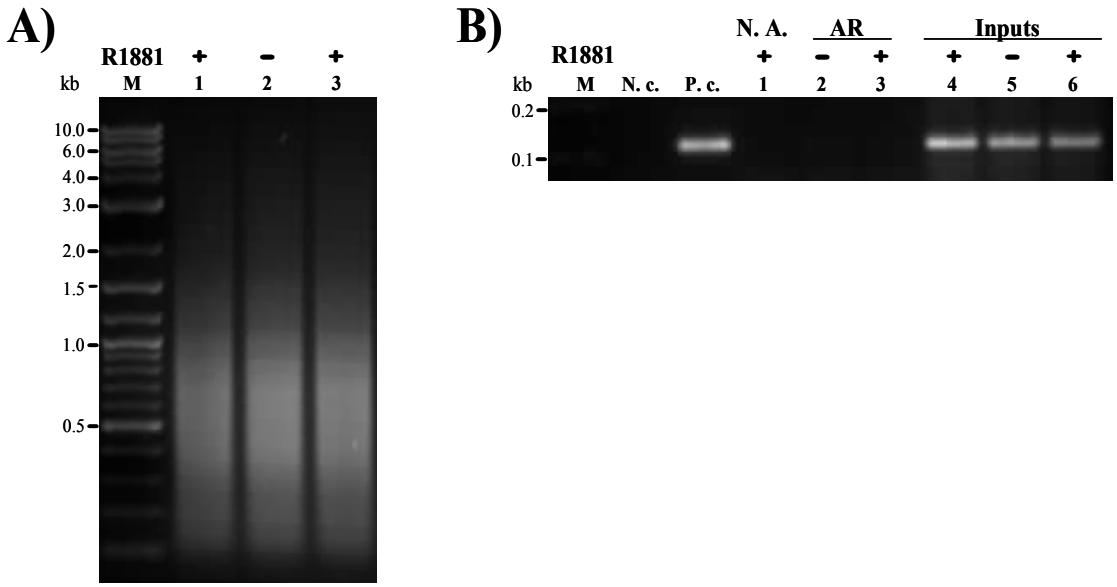


Figure 4. ChIP assay to assess AR binding to its HREs in the MMTV promoter using standard protocol conditions

3108 cells were left untreated (-) or treated (+) with R1881 (10^{-8}M) for 1 h and subjected to the ChIP assay using an antibody specific to AR, as described in Materials and Methods. The DNA molecular weight markers (M; in kb) are shown at the left of the figures. (A) Sheared DNA, (B) ChIP assay performed with AR antibody (2µg). No antibody (N.A.) was used as negative background control. Inputs represent the starting material before immunoprecipitation. Negative (N.c.) and positive (P.c.) are PCR performed in the absence of DNA containing the MMTV promoter, respectively.

Since the PCR controls and inputs showed expected results, we reasoned that it may be the immunoprecipitation or the following steps that failed during the experiment. This could have been due to inefficient crosslinking, unsuccessful binding to the beads, incomplete/lack of elution or problems with the antibody. Thus, we first checked crosslinking conditions to assess if it was functional. Crosslinking is a time critical procedure, depending on the cell type and protein of interest. Excessive crosslinking can lead to decrease in the amount of protein bound to DNA and to reduction in the availability of epitopes for antibody binding, while too little crosslinking may not fix protein to its chromatin binding site. In the experiment presented above, crosslinking was carried out for 10 min at RT, but no signal was detected. Thus it became of interest to increase crosslinking time to see if this can

help. As can be seen in Figure 5A, even longer crosslinking time did not improve the signal obtained in ChIP. In addition, crosslinking was also performed on ice for 20 and 30 min (Figure 5B) which either did not help either.

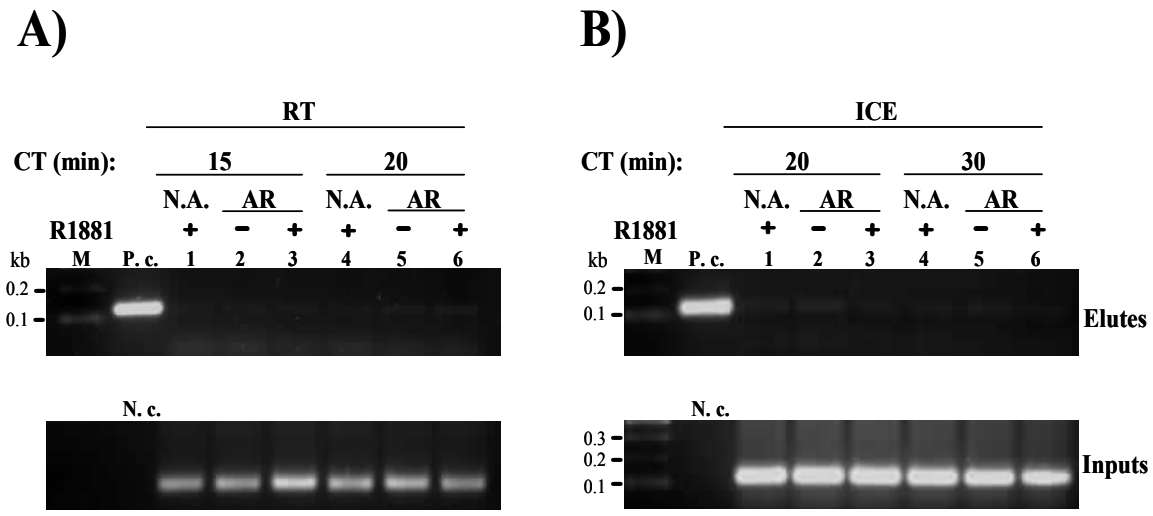


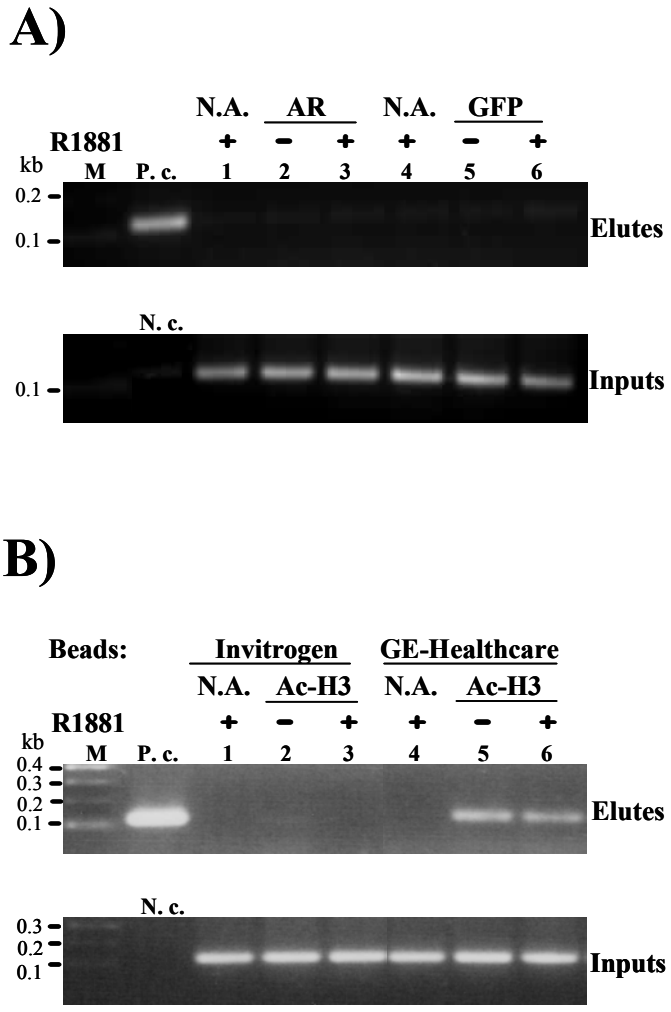
Figure 5. ChIP assay, optimization of crosslinking

3108 cells were untreated (-) or treated (+) with androgen R1881 (10^{-8}M) for 1 h and subjected to the ChIP assay using an antibody specific to AR, as described in Materials and Methods. The DNA molecular weight markers (M; in kb) are shown at the left of the figures. No antibody (N.A.) was used as negative background control. Inputs represent the starting material before immunoprecipitation. Negative (N.c.) and positive (P.c.) PCR controls are indicated. (A) ChIP assay with crosslinking time (CT) of 15 and 20 min at room temperature (RT). (B) ChIP assay with crosslinking time (CT) of 20 and 30 min on ice.

Having shown that crosslinking was not a problem, the next step was to check if use of different antibodies specific for GFP-AR would improve the signal of precipitated samples. Antibodies are used to capture the DNA/protein complex and this step is typically the most critical one for a successful ChIP experiment. Thus, the antibody specific to AR (N-20, Santa Cruz) that was used in the experiments presented above for immunoprecipitation was changed with another AR-specific antibody (Upstate)

and an antibody specific to GFP (Invitrogen). However, neither of these antibodies resulted in a signal similar to experiments above (Figure 6A).

Since varying the antibody did not help to solve the problem, we then turned to the Protein A Sepharose beads that are necessary for successful immunoprecipitation. In previous experiments we used Protein A Sepharose beads obtained from Invitrogen. To examine if there was something wrong with these beads, the next ChIP was performed with beads from a different supplier (GE-Healthcare) side by side with those from Invitrogen (Figure 6B).



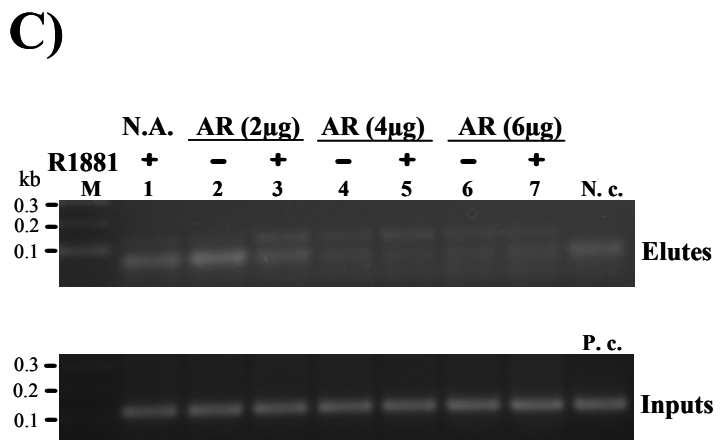


Figure 6. Optimization of the ChIP assay with different antibodies and beads

3108 cells were untreated (-) or treated (+) with androgen R1881 (10^{-8}M) for 1 h and subjected to ChIP assay, as described in Materials and Methods. The DNA molecular weight markers (M; in kb) are shown at the left of the figures. No antibody (N.A.) was used as negative background control. Inputs represent the starting material before immunoprecipitation. Negative (N.c.) and positive (P.c.) PCR controls are indicated. (A) ChIP assay performed with antibodies specific to AR (Upstate) and GFP (Invitrogen). (B) ChIP assay performed with Protein A Sepharose (Invitrogen) and (GE-Healthcare), and acetyl-histone H3 antibody. (C) ChIP assay performed with different amounts of AR (Santa Cruz) antibody, respectively 2, 4 and 6 µg.

We used an antibody for acetyl-histone H3 which was previously shown to function well in ChIP (data not shown); since histones are far more abundant than chromatin bound AR, we expected to see a better signal if the ChIP failure was not due to a bead problem. As can be seen in Figure 6B, there is an expected signal only in samples that were processed with Protein A Sepharose obtained from GE-Healthcare. The Sepharose beads that were used in previous experiments resulted in a very weak band in the sample without hormone treatment (Figure 6B, lane 2). These results indicate that the failure of the ChIP assays presented previously was due to a problem with the Protein A Sepharose beads.

As Protein A Sepharose beads were shown to be the cause of previous ChIP failures, we performed ChIP using new Protein A Sepharose beads and the AR antibody (used in Figure 4 and 5), (Figure 6C). In addition, a titration of the AR antibody was carried out to determine the optimal amount to use. Figure 6C shows that 2µg of AR

antibody gave the best result, considering the ligand effect (lanes 2 and 3), compared to other concentrations that were used. With increasing amount of the antibody, there was a parallel increase in the background signal which diminished the ligand effect that was observed.

Taken together, these results document that new Protein A Sepharose beads and the same amount of antibody as used in the standard protocol result in a proper ChIP signal. However, the signal is still weak and need further optimization, possibly by trying different antibodies and/or changing protocol conditions.

4.5 Effect of TSA on histone acetylation at the MMTV promoter in the presence of AR

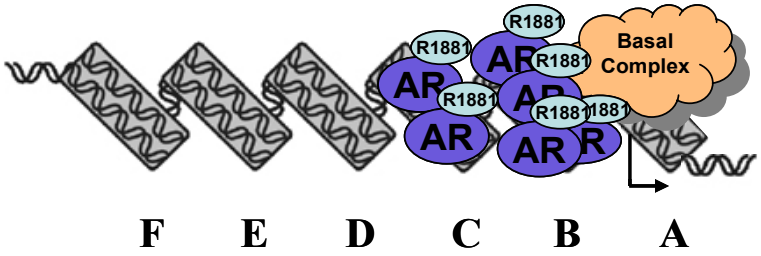
Although TSA treatment induces an increase in global histone acetylation and modulates the activity of various promoters, it is often not clear whether there are changes in histone acetylation at specific target promoters. To investigate the effect of TSA on acetylation of histones at the MMTV promoter in response to AR activation, 3108 cells were either left untreated or treated with R1881, or TSA, alone or in combination, and ChIP assays were carried out with antibodies specific to the acetylated form of histone H3 and H4 (Figure 7). The MMTV promoter has six well defined positioned nucleosomes (Nuc) termed A-F (Richard-Foy and Hager, 1987). Nuc-B contains most of the HREs or and was subject of primary interest in this study (Figure 7A). To that end, the analysis of ChIP experiments was performed by PCR with primers specific to the Nuc-B region of the MMTV promoter. A representative analysis is shown in Figure 7B.

There is a relatively high basal acetylation level of both histone H3 and H4 prior to treatment with R1881 and TSA (lanes 4 and 8). When the cells were treated with TSA alone, no change in acetylation of histone H3 (lane 6) and a moderate increase in acetylation of histone H4 (lane 10) was observed, compared to untreated cells. Surprisingly, androgen treatment reduced the basal acetylation level of histone H3

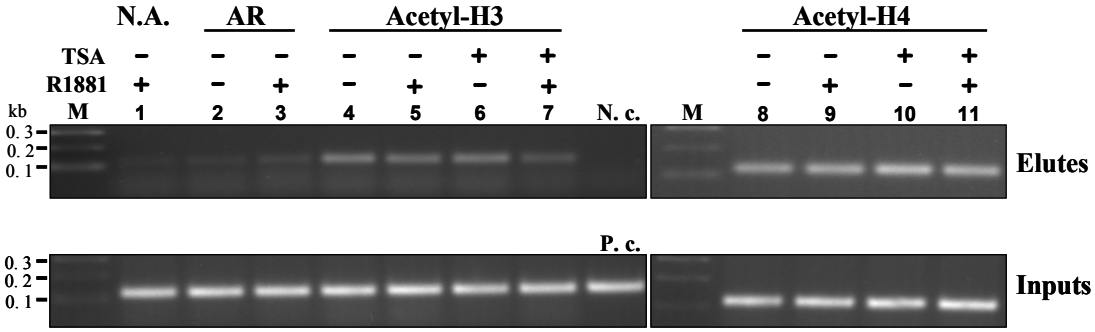
(lane 5), which further decreased in combination with TSA (lane 7). However, acetylation of histone H4 remained relatively unchanged upon hormone treatment, compared to that observed for untreated cells (lanes 9 and 11).

The analysis of the ChIP experiments was also performed by quantitative PCR (qPCR) and a graphic summary of three independent experiments is shown in Figure 7C.

A)



B)



C)

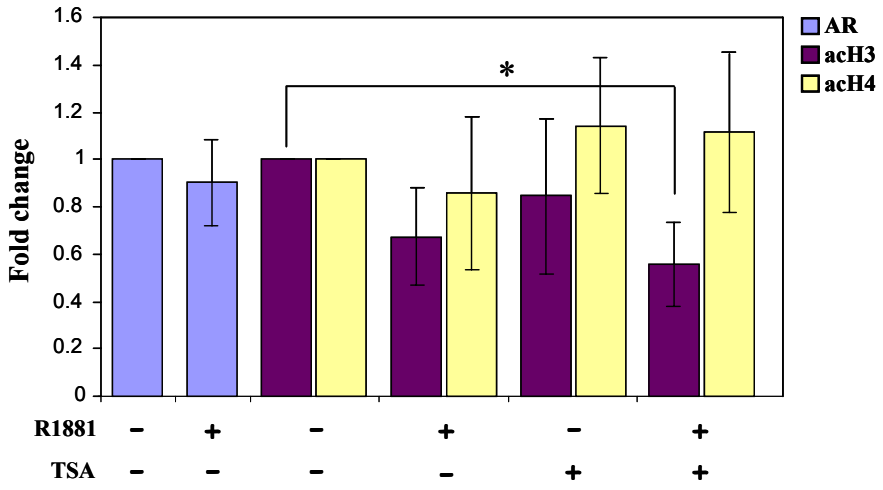


Figure 7. Effect of TSA on acetylation of histone H3 and H4 at MMTV promoter nucleosome B in the presence and absence of R1881

3108 cells were left untreated or were treated with R1881 (10^{-8}M) for 1 h, treated with TSA (100nM) for 18 h, or pretreated with TSA (100nM) for 17 h followed by R1881 (10^{-8}M) for 1 h. ChIP assays were performed as described in Materials and Methods, using antibodies against AR or acetylated histone H3 or H4. (A) A schematic presentation of the MMTV promoter. The approximate positions of the nucleosomes (A-F) as well as the binding sites for AR and basal transcription factors are indicated. The arrow shows the transcription start site. (B) A representative ChIP assay. Inputs represent the starting material before immunoprecipitation. No antibody (N.A.) was used as negative background control. Negative (N.c.) and positive (P.c.) PCR controls are indicated. The DNA molecular weight markers (M; in kb) are shown at the left of the figure. (C) The graph represents quantification of the real-time PCR results. ChIP signals determined by real-time PCR were normalized with the corresponding inputs and normalized-untreated samples were set to 1. Values mark the mean of three independent experiments with the error bars representing standard deviation. * means $p < 0.05$ as determined by Student's t-test.

The qPCR results showed a decrease in acetylation of histone H3 in the presence of androgen R1881, which is consistent with observations in Figure 7B. In fact, combination of hormone and TSA treatment resulted in a significant loss of histone H3 acetylation to about 50% of that seen in untreated cells. Furthermore, the presence of hormone appeared to decrease acetylation of histone H4, which increased when combined with TSA treatment. This does not correspond with the results observed in Figure 7B. Additionally, TSA treatment in the absence of R1881 reduced histone H3

and increased histone H4 acetylation which also differs from previous observations. Overall, these results indicate that acetylation of histone H3 in the Nuc-B region of the MMTV promoter is not increased by TSA under any treatment condition and is reduced upon R1881 treatment whereas there are no significant changes in acetylation of histone H4 under these conditions.

In order to assess R1881-induced binding of AR with its binding sites at Nuc-B region of the MMTV promoter, ChIP experiments were also performed with antibody specific to AR. However, there was only a small increase in AR binding to HREs upon androgen activation observed, compared to untreated cells (Figure 7B, lanes 2 and 3). In fact, qPCR analysis revealed that AR enrichment level in the presence of androgen is somewhat lower than that observed in untreated cells (Figure 7C). Furthermore, ChIP experiments showed a weak background signal in no antibody control, as expected (Figure 7B, lane 1). These results again indicate that the ChIP experiments for AR need to be optimized further.

4.6 Effect of TSA on histone acetylation at the MMTV promoter in the presence of GR

The glucocorticoid receptor (GR), a member of steroid hormone receptor family, is closely related to the AR. Previously, it has been demonstrated that GR and AR bind in a similar dynamic manner to the same HREs of the MMTV promoter (McNally et al., 2000; Klokk et al., 2007). However, in response to HDACis, GR has been reported to possess a different behavior at the MMTV promoter compared with the AR. It was shown that, in contrast to AR, TSA inhibits GR transcriptional activity and does not affect the mobility of the agonist bound GR on the MMTV promoter (Mulholland et al., 2003; Saatcioglu lab, unpublished data). Therefore it became of interest to investigate the effect of TSA on acetylation of histones at the MMTV promoter in response to GR activation. ChIP assays were performed with antibodies specific to the acetylated form of histone H3 and H4 in 3617 cells in the presence or

absence of Dex/TSA (Figure 8). As GR binds to the same HREs of the MMTV promoter as AR (see Figure 7A), Nuc-B contains most of the GR-binding sites. The analysis of ChIP experiments were carried out by PCR with primers specific to the Nuc-B region of the MMTV promoter similar to AR. A representative analysis is shown in Figure 8A. There was an expected increase in GR binding to HREs upon Dex activation compared to untreated cells (lanes 2 and 3). This was consistent with the results obtained by qPCR analysis. There was high level of basal acetylation of both histone H3 and H4 prior to treatment with Dex and TSA (lanes 4 and 8), similar to that observed in 3108 cells for AR. Furthermore, histone H3 showed relatively similar acetylation levels under all treatment conditions that were lower than basal acetylation (lanes 5-7). However, acetylation of histone H4 remained relatively unchanged upon hormone and TSA treatment alone, compared to untreated cells (lanes 9 and 10), with a small increase observed upon combination of these two treatments (lane 11).

The analysis of the ChIP experiments was also performed by qPCR and a graphic summary is shown in Figure 8B. The graph presented in Figure 8B represents results obtained from one single ChIP experiment; additional experiments are in progress. The qPCR results showed a small decrease in acetylation of histone H3 in the presence of Dex alone and in combination with TSA, which is consistent with observations in Figure 8B. Additionally, acetylation of histone H4 was decreased in the presence of TSA alone and in combination with Dex, and increased upon hormone treatment. These results do not correspond with results observed in Figure 8B that show relatively unchanged levels of histone H4 acetylation, which indicates the need for additional experiments.

Taken together, TSA treatment does not appear to increase acetylation levels of histone H3 and H4 in the Nuc-B region of the MMTV promoter of 3617 cells and it does not seem to be reduction in H3 acetylation upon Dex treatment. Thus, there may be differences between AR- and GR-mediated changes in histone acetylation at the MMTV promoter.

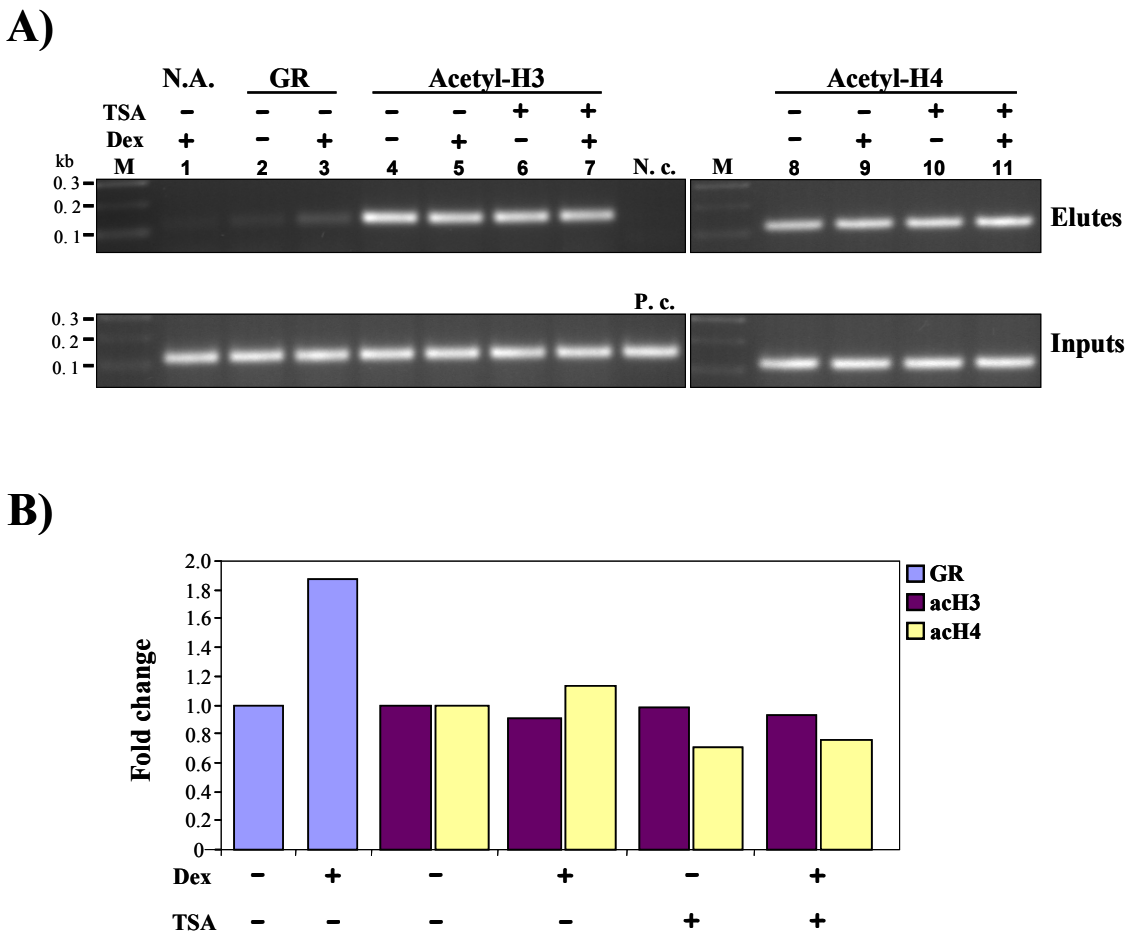


Figure 8. Effect of TSA on acetylation of histone H3 and H4 at MMTV promoter nucleosome B in the presence and absence of Dex

3617 cells were treated with Dex (10^{-7}M) for 30 min or TSA (100nM) for 18 h, alone or in combination or were left untreated. ChIP assays were performed as described in Materials and Methods, using antibodies against GR or acetylated histone H3 or H4. (A) A representative analysis of ChIP assay. Inputs represent the starting material before immunoprecipitation. No antibody (N.A.) was used as negative background control. Negative (N.c.) and positive (P.c.) PCR controls are indicated. The DNA molecular weight markers (M; in kb) are shown at the left of the figure. (B) The graph represents quantification of the real-time PCR results. ChIP signals determined by real-time PCR were normalized with the corresponding inputs and normalized-untreated samples were set to 1. Values represent results of one single experimen

5. Discussion and Future Perspectives

Histone acetylation represents an important mechanism in the regulation of gene activity. Acetylation is generally associated with increased gene transcription whereas deacetylation is linked to transcriptional repression (Ng and Bird, 2000; Berger, 2002; Verdone et al., 2005). However, a number of studies suggest a more complex scenario with respect to individual genes and/or promoters (Deckert and Struhl, 2001; Mulholland et al., 2003) and the underlying mechanisms are not fully understood.

Previous work in our laboratory demonstrated the consequences of changes in histone acetylation induced by the HDAC inhibitor TSA on dynamics and transcriptional activity mediated by AR and GR at the MMTV promoter (unpublished data). While TSA reduced AR mobility that correlated with an increase in transcriptional activity, the mobility and transcriptional activity of GR was not affected by TSA. These observations were consistent with previous investigations that reported differential response of AR and GR to TSA at the MMTV promoter (List et al., 1999; Mulholland et al., 2003).

In this study, the acetylation status of the MMTV promoter in response to the HDAC inhibitor TSA in the presence of AR and GR was examined with the aim to get clues as to why these two receptors respond differently to TSA. The data presented indicate that there are some differences in acetylation levels of histone H3 at the MMTV promoter in response to TSA in the presence of AR, but not GR (Figure 7B and 8A). The treatment with AR-agonist R1881, independent of the presence of TSA, resulted in a decrease in histone H3 acetylation at Nuc-B within the MMTV promoter (Figure 7B, lane 5), which was further decreased after TSA treatment (Figure 7B, lane 7). These observations indicate an unexpected negative correlation between MMTV transcriptional activation by AR reported previously (List et al., 1999; Klokk et al., 2007), and histone acetylation at the MMTV promoter. These data suggest that the increased transcriptional activity of AR at the MMTV promoter in response to TSA is associated with another mechanism than increased histone acetylation. Results

obtained from preliminary studies of the MMTV promoter in the presence of GR suggest a different pattern of histone H3 acetylation compared to AR (Figure 8B). The acetylation level of histone H3 is relatively unchanged after treatment with the synthetic glucocorticoid Dex alone or in combination with TSA; this is correlated with no effect of TSA on GR transcriptional activity at the MMTV promoter. However, it should be pointed out that additional studies, including replication of the experiments presented here, are needed in order to draw final conclusions regarding the details of histone acetylation during AR- and GR-mediated transcriptional activation at the MMTV promoter in response to TSA. The variations that we experienced in the different ChIP experiments could be due to variations between batches of antibody, the chromatin preparation, or differences in the efficiency of the actual immunoprecipitation process for a given antibody; further work is in progress to check these possibilities.

We found that in any of the ChIP analyses of histone H3 or H4 at Nuc-B of MMTV promoter, under any treatment conditions, there was no increased acetylation (Figure 7B and 8A, lanes 6, 7, 10 and 11). Thus, even though TSA treatment induced global histone hyperacetylation (Figure 3A and 3B), the MMTV promoter, at least the Nuc-B region, was unaffected. Together**,** these results suggest that the primary target of HDAC inhibition by TSA at the MMTV promoter may be a non-histone coregulatory protein that is involved in an essential step of AR- or GR-mediated transcription rather than core histones. Alternatively, dynamic acetylation and deacetylation of specific residues of histone H3 and H4 may be occurring that cannot be detected in a global search for changes.

Potential non-histone targets of TSA could be HDACs, which have shown to possess both activation and repression properties as coregulators of nuclear receptors (for a review, see Gallinari et al., 2007). For instance, if TSA inhibited the repressive function of an AR-associated HDAC and activation action of a GR-associated HDAC, it could potentially explain opposite effect of the same compound. HDAC1 is indeed sensitive to inhibition by TSA (Marks et al., 2001) and have a distinct role in

transactivation by AR and GR (Shang et al., 2002; Qiu et al., 2006). While HDAC1 had an inhibitory effect on AR activity on the PSA promoter, suggesting that it acts as a corepressor for AR (Shang et al., 2002), it was shown to serve as a coactivator for GR-mediated activation of the MMTV promoter (Qiu et al., 2006). Interestingly, the coactivator activity of HDAC1 was shown to be regulated by acetylation that suppresses its deacetylase activity, allowing GR-induced transcription (Qiu et al., 2006). It would therefore be of interest to study the acetylation status of HDAC1 and its association with both receptors under the conditions used in this study.

AR and GR are also subject to posttranslational modifications that play a role in modulation of their transcriptional activities (reviewed in Faus and Haendler, 2006). Among these, both AR and GR have acetylation motifs (KxKK) within their DBD-hinge domains (Fu et al., 2000; Ito et al., 2006). Hormone dependent activation of AR was shown to be dependent on acetylation of three lysine residues in this region by coactivators that harbour HAT activity and modulate AR transcriptional activity by favouring nuclear translocation and altering its association with coactivators and corepressors (Fu et al., 2000; Fu et al., 2002; Gaughan et al., 2002). GR, similar to AR, is also acetylated at this motif upon hormone activation, although this does not seem to affect its transcriptional activity (Ito et al., 2006). Thus, TSA treatment may inhibit HDACs that deacetylate both AR and GR, resulting in increased acetylation that would possibly increase AR activity without effecting function of GR. Indeed, earlier studies have demonstrated that TSA treatment results in increased acetylation, and thereby increased activity, of AR (Fu et al., 2000; Fu et al., 2003). Even though AR acetylation may explain its increased activity in response to HDAC inhibition, there is also a possibility of other modifications or cross-talk between the modifications being involved. For example, AR is also known to be a phosphoprotein (Gioeli et al., 2002); there could potentially be interactions between acetylation and phosphorylation events that determine AR activity. Exploring the acetylation status of AR/GR and use of acetylation mutants may further reveal if the different responses of

AR and GR to TSA are connected to their posttranslational modification by acetylation.

An alternative explanation of the current results is that inhibition of HDAC activity could lead to increased acetylation of a member of the transcription initiation complex, such as general transcription factors or coactivators that could account for increased/decreased transcriptional activity. In fact several coactivators that interact with the basal transcription machinery, including PCAF, p300, and SRC1 are known to be acetylated (Chen et al., 1999; Sterner and Berger, 2000) and these act as cofactors for AR and GR (for reviews, see Jenkins et al., 2001; Heinlein and Chang, 2002). However, the effect of acetylation on the function of these proteins is currently unknown. Alternatively, it is possible that an HDAC involved in GR-mediated transcription would be insensitive to TSA treatment, resulting in a different response than with AR.

It is important to note that in the studies described in this thesis, the cells were exposed to TSA for relatively long periods of time (18 hours). During this time period, some secondary effects of HDAC inhibition could occur, such as changes in expression levels of AR or GR, or their coregulatory proteins. Recent data from our laboratory indicate that there is an increase in expression of both AR and GR in response to TSA (unpublished data). However, it is not clear whether these changes alone can account for the transcriptional effects observed. In addition, expression levels of HDAC1 in response to TSA and corresponding ligands were also examined by Western analysis and immunostaining. The results showed no change in expression levels of HDAC1 in the presence of TSA and androgen or glucocorticoid (Saatcioglu laboratory, unpublished results). Thus, different effect of TSA on AR- and GR-mediated transcription is most probably not connected to differences in expression levels of the receptors or HDAC1.

Overall, although ChIP analyses indicate differences in the acetylation profile of the MMTV promoter in the presence of AR compared to GR in response to TSA, these do not seem to directly correlate with AR and GR transcriptional activity, suggesting

a more complex mechanism of transcriptional regulation. It is possible that increased acetylation of non-histone proteins may have equal or greater importance in mediating effects of TSA. However, as pointed out above, more direct and significant changes in the acetylation status of selected sites on histones may occur which could be critical for the observed differences between AR and GR. Further work is required to examine these possibilities.

Finally, it should be emphasized that although ChIP analysis is a powerful method used to examine chromatin-related processes and was the primary method used in this study, additional optimization is required for some of the experiments. For example, although ChIP conditions were extensively optimized prior to the experiments, only a small difference could be observed in the enrichment of AR on the MMTV promoter in response to androgen. We now know that this is due to the limitation of the AR antibody that we used, as most recent studies using GFP antibody reproducibly give significant enrichment of MMTV-bound AR in response to hormone (Saatcioglu laboratory, unpublished results). Thus, some of the experiments presented in this thesis need to be repeated for conclusive results.

In order to determine what accounts for differential responses of AR and GR to TSA on the MMTV promoter, it is important to identify the exact factors that are used in transcriptional activation by the two receptors in this context. For example, it is of interest to determine which HDACs are involved with each receptor and how they affect their transactivation potential on the MMTV promoter. As briefly discussed above, one obvious candidate in this regard is HDAC1 whose possible contributions could be checked through overexpression and/or knock-down experiments. Furthermore, it may be essential to examine the acetylation status of other nucleosomes at the MMTV promoter upon hormone and TSA treatment, and also investigate the acetylation status of specific residues within each histone. In addition, the investigation of AR acetylation mutants would conclusively assess whether AR acetylation is involved in its response to TSA. These and other studies will shed light on the details of AR/GR-chromatin interplay at the MMTV promoter.

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